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# **Characterisation of dendritic cell subsets in inflammatory arthritis**

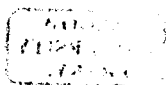
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A thesis submitted to the Faculty of Medicine, University of Glasgow for the degree of  
Doctor by Philosophy

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## Summary

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are chronic inflammatory arthropathies of heterogeneous aetiology affecting 2-3% of the general population. Extensive *in vivo* and *in vitro* studies have identified the multiple pro-inflammatory cytokines and enzymes implicated in the pathogenesis of these disorders culminating in therapeutically beneficial cytokine targeted therapies. Cytokine blockade however exhibits variable responses across patient populations and importantly disease activity recurs upon cessation of therapy. The critical challenge in therapeutics now is to uncover the underlying immunological processes responsible for the generation and perpetuation of inflammatory arthritis such that peripheral tolerance can be re-established and therapeutic remission can be maintained. Of particular interest are the co-stimulatory interactions bridging the innate and adaptive immune response, notably those initiated by the interaction of dendritic cells (DC) with T cells, as the evolution of the latter is profoundly influenced by these cognate interactions. Moreover, DC are the only APC recognized to date as capable of provoking autoimmune disease and therefore embody an important line of investigation in determining the initial triggers that generate and (of therapeutic significance) maintain chronic inflammatory arthritis. However, to date there have been very few investigations of the phenotype and function of DC, in particular with reference to DC lineage. This partly reflects difficulty in adequately identifying the DC subsets, plasmacytoid (p) DC and myeloid (m) DC, in peripheral blood and tissues – most methodologies rely on complex combinations of antibody markers. Recently, novel markers useful in human, and murine, DC studies have been defined that can improve resolution of DC subset heterogeneity. This study therefore focused on utilizing novel DC markers in a combination of *in vitro* and *in vivo* analyses in order to elucidate the contribution of different DC subsets to the initiation, modulation and perpetuation of the inflammatory arthritides, rheumatoid arthritis (RA) and psoriatic arthritis (PsA).

The first part of this thesis examined by comparative analysis the phenotype, tissue distribution and functional profile of the human DC subsets, myeloid (m) DC and plasmacytoid (DC), in RA and PsA. This study demonstrated that circulating pDC and mDC are reduced in the peripheral blood (PB) of RA and PsA patients, but are accumulated to the inflammatory synovial fluid (SF) and synovial membrane (SM), commensurate with altered migration. Furthermore, it was demonstrated that the majority

of pDC and mDC within the synovial compartment displayed a predominantly immature phenotype as identified by the low to absent expression of specific co-stimulation and maturation markers including CD80, CD83, CD86 and dendritic cell lysosome-associated membrane protein (DC-LAMP). However, pDC and mDC purified from SF underwent *ex vivo* phenotypic maturation and released cytokines to TLR stimulation at comparable levels to their normal PB purified counterparts. Commensurate with that observation, cytokine profiling of SM localized pDC and mDC revealed expression of the pro-inflammatory cytokines IL-12p70, IL-23p19, IL-15, IL-18 and IFN- $\alpha/\beta$ . Together these data indicated no apparent intrinsic cellular defect, suggesting that extrinsic factors in the local synovial environment may be culpable for inhibition of DC maturation. In addition mDC in SM expressed predominantly IL-12p70 and IL-23p19, suggesting a central role in the regulation and expansion of T cells, in particular Th17, which have been ascribed a pathologic role in arthritis. In contrast, pDC expressed predominantly IL-15 and IL-18, cytokines that can enhance IL-12 induced IFN- $\gamma$  release and inhibit T cell apoptosis, thus indicating a role in the amplification and prolongation of inflammatory arthritis. Of particular interest it was demonstrated that pDC also expressed IFN- $\alpha/\beta$  in abundance. As pDC constituted the dominant DC subset in the SM in both RA and PsA, this may have pathological implications as IFN- $\alpha$  has been critically linked to pathology in other autoimmune diseases, including systemic lupus erythematosus (SLE) and, of particular import to PsA, psoriasis.

Thereafter I aimed to elucidate the effect of cytokine modification on the function of mDC and pDC in regard to synovial inflammation, and the resolution thereof, to gain further insight into the contribution of DC to disease pathology. Specifically, we examined DC subsets in synovium after TNF- $\alpha$  blockade (infliximab) in RA and PsA patients and compared and thereafter contrasted the effects of both of TNF- $\alpha$  and IL-15 on DC subsets in *in vitro* assays. While this component of the study was hampered by cell rarity, low number of participant patients and the early time point examined after infliximab infusion, it indicated a number of potentially informative trends. Pre-eminent amongst these was the observation that TNF blockade may have differential effects on mDC and pDC. In particular, we observed a trend towards decreased mDC in RA and PsA synovium 48 hours after infliximab infusion. Consistent with this, TNF neutralization down-regulated mDC release of the pro-inflammatory chemokines CCL2, CCL4 and CXCL8 in *in vitro*



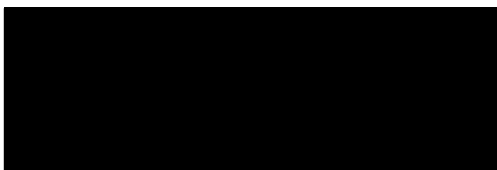
experiments of mDC/T cell contact. In contrast, pDC were neither decreased in synovium after infliximab infusion nor did TNF neutralization reduce chemokine release in pDC/T cell contact. Indeed, TNF neutralization appeared to *increase* the release of pro-inflammatory chemokines from pDC/T cell contact, which may be explained by IFN- $\alpha$  which has been demonstrated to drive CCL2, CCL3 and CCL4 secretion from pDC, but not mDC. Together the data suggest the intriguing possibility that TNF blockade could actually enhance pDC arthritogenicity, presenting a potential explanation as to why some patients fail TNF therapy. We also observed that these effects were apparently TNF specific, since IL-15 neutralisation appeared to decrease, TNF- $\alpha$  and pro-inflammatory chemokine production from both mDC/T cell and pDC/T cell contacts. Our *in vitro* analyses of the biological action of IL-15 suggested that this may be attributable to the direct inhibition of DC cognate interactions with T cells by IL-15 blockade via down-regulation of CD69 and inhibition of IL-2 enabled T cell expansion. As such IL-15 blockade may offer an appealing complementary therapy to TNF directed therapy.

Finally, this study sought to compare the functional role of mDC and pDC in the OVA TcR transgenic T cell adoptive transfer model of arthritis by means of depletion of the former using pDC specific antibodies. This study revealed that depletion of pDC at the time of OVA challenge increased the histopathological severity, serum autoantibody concentration and (auto) antigen-specific T cell re-stimulation significantly, as such demonstrating that pDC may be critical to the suppression of autoimmunity, by (presumably) induction of peripheral tolerance. By association, these results also suggested that mDC were capable of inducing inflammatory arthritic autoimmunity in the absence of pDC.

In conclusion, these studies indicate that mDC and pDC possess multi-faceted roles in inflammatory arthritis, involved in both the initiation and perpetuation of arthritis, but also the potential suppression thereof. The data provide a firm basis upon which to build rational tolerance induction regimes in due course as the appropriate biological targeting agents become available in the clinical environment.

## **Declaration**

I declare that the following thesis has been composed by myself, that it embodies the results of my own special work, and that it does not include work forming part of a thesis presented successfully for a degree at this or any other university.



**Sarah Louise Jongbloed**

**August 2006**

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## Abbreviations

Ab	antibody
ACR	American College of Rheumatology
Ag	antigen
AkS	Ankylosing spondylitis
APC	antigen-presenting cell
ARA	American Rheumatism Association
BDCA	blood dendritic cell antigen
BiP	immunoglobulin binding protein
BM	bone marrow
BSA	bovine serum albumin
CII	collagen type II
CARD15	caspase recruitment domain-containing protein-15
CCP	cyclic citrullinated protein
CCR	chemokine receptor
CIA	collagen-induced arthritis
CMV	cytomegalovirus
CpG	immunostimulatory bacterial CpG-DNA sequence
CD	cluster of differentiation
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CMKR1/ChemR23	chemokine-like receptor 1
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
COX-2	cyclo-oxygenase type 2
CTLA	cytotoxic T-lymphocyte associated antigen
DAB	3,3'-diaminobenzidine
DC	dendritic cell
DC-LAMP	dendritic cell lysosome-associated membrane protein
DMARDS	disease modifying anti-rheumatic drugs
DNA	deoxyribonucleic acid
DC-SIGN	dendritic cell-specific ICAM3-grabbing non-integrin

EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-barr virus
EGF	epidermal growth factor
eIF4GI	citrullinated eukaryotic translational initiation factor 4GI
ELISA	enzyme-linked immunosorbance assay
ELC	Epstein–Barr virus-induced receptor ligand
ERK	extracellular regulated kinase
FACS	fluorescence-activated immunosorbant assay
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FLS	fibroblast-like synoviocyte
FLT-3L	fms-like tyrosine kinase 3-ligand
Foxp3	forkhead-box p3
FSC	forward scatter
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glucose-6-phosphate isomerase
h	hours
HBSS	Hanks' buffered salt solution
HCgp-39	human cartilage gp-39
HEV	high endothelial venule
HLA	human leukocyte antigen
hnRNP-A1	heterogeneous nuclear ribonucleoprotein-A1
HIV	human immunodeficiency virus
ICAM	intracellular adhesion molecule
IDO	indoleamine 2,3-deoxygenase
IFN	interferon
Ig	immunoglobulin
IGF	insulin-like growth factor
IL	Interleukin
ILAR	International league against rheumatism
ILT	immunoglobulin-like transcript
iNOS	inducible oxide synthase

IOD	integrated optical density
i.p	intraperitoneal
IP-10	IFN- $\gamma$ inducible protein
IRAK	IL-Ra-associated kinase
i.v	intravenous
JAK	janus kinase
JNK	c-Jun N-terminal kinase
K13	keratin 13
kD	kilo Daltons
KGF	hepatocyte growth factor
LARC	liver and activation-related chemokine
LC	Langerhans cell
LCA	leukocyte common antigen
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAP	mitogen activated protein
MCP	monocyte chemoattractant protein
M-CSF	macrophage-colony stimulating factor
mDC	myeloid DC
MDC	macrophage-derived chemokine
MHC	major histocompatibility complex
MICA	MHC class I chain-related gene A
MIF	macrophage inhibitory factor
MIP	macrophage inflammatory protein
moDC	monocyte derived DC
MMP	matrix metalloproteinase
mRNA	messenger RNA
NFAT	nuclear factor of activated T cells
NF- $\kappa$ B	nuclear factor- $\kappa$ B
NK	natural killer
NO	nitric oxide
NOD	nucleotide-binding oligomerisation domain

NSAIDS	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
OD	optical density
ODN	oligodeoxynucleotide
OVA	Ovalbumin
PADI4	peptidylarginine deiminase type 4
PAMP	pathogen-associated molecular patterns
PARC	pulmonary and activation-regulated chemokine
PB	peripheral blood
PBS	phosphate buffered saline
pDC	plasmacytoid DC
PDCD-1	programmed death receptor-1
PDGF	platelet derived growth factor
PE	phycoerythrin
PGE-2	prostaglandin-E2
PI	propidium iodide
PI-3K	phosphoinositide 3-kinase
PKC	protein kinase C
PsA	psoriatic arthritis
PTK	protein tyrosine kinase
PTPN22	protein tyrosine phosphatase N22
PUFA	polyunsaturated fatty acid
RA	rheumatoid arthritis
RA33	ribonucleoprotein A2
RAB11FIP1	rab coupling protein isoform 3
RANK-L	receptor activator of nuclear factor $\kappa$ B ligand
RANTES	regulated on activation normal T cell expressed and secreted
RF	rheumatoid factor
RNA	ribonucleic acid
SA	streptavidin
SEM	standard error of the mean
s.c	subcutaneous
SF	synovial fluid
SLC	secondary lymphoid tissue chemokine

SLE	systemic lupus erythematosus
SM	synovial membrane
SNP	single-nucleotide polymorphism
SOCS	suppressor of cytokines
SSC	side scatter
TARC	thymus stromal lymphopoeitin
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
TLR	toll-like receptor
TNF	tumour necrosis factor
Treg	regulatory T cell
TLSLP	thymic stromal lymphopoeitin
TRANCE	tumour necrosis factor-related activation induced cytokine
UDPGD	uridine diphosphoglucose dehydrogenase
UK	United Kingdom
USA	United States of America
VCAM	vascular adhesion molecule
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

**Chapter 1: Introduction**

## **1.1 Inflammatory Arthritis: Rheumatoid Arthritis and Psoriatic Arthritis**

The inflammatory arthritides comprise a heterogeneous group of autoimmune disorders characterised by chronic joint inflammation, immune cell infiltration to the synovium, fibroblast-like synoviocyte (FLS) expansion and irreversible destruction of cartilage and bone. A source of significant health care expenditure worldwide, arthritis affects 2-3% of the general population and is projected to affect 67 million people by 2030 in the United States of America (USA) alone (1-3). Rheumatoid arthritis (RA) is the most prevalent of the chronic inflammatory arthropathies affecting 0.5-1.0% of the general population (4, 5). Psoriatic arthritis (PsA) is the second most common inflammatory arthritis (6). Defined as a (predominantly) seronegative arthritis associated with psoriasis, the exact prevalence of PsA is unknown due to a lack of specific diagnostic criteria, but is estimated at 0.3-1.0% of the general population and 6-42% of all psoriasis sufferers (7-10). While RA has been the focus of intensive clinical, immunological and genetic investigations, PsA has been comparatively less well defined, perhaps in part because it was only recognised as a distinct disease entity in 1964 (11). PsA was originally considered by many to share similar if not identical pathogenesis mechanisms with RA. Subsequent analyses however have indicated a stronger association of PsA with the spondyloarthropathies, including ankylosing spondylitis (AS), enteropathic arthritis and reactive arthritis (12). It is now widely accepted that both RA and PsA are distinct diseases, arising from multifactorial conditions. Whereas epidemiological studies have highlighted numerous genetic and environmental risk factors associated with disease occurrence and expression, the immunological mechanisms that initiate and perpetuate these diseases remain unclear. As a consequence the pursuit of novel therapies is impaired.

### **1.1.1 History**

The earliest evidence of RA comes from paleopathological analysis of 6500 year old skeletons, discovered near the Tennessee and Green rivers in the USA, displaying a polyarticular, symmetrical erosive arthritis with no sacroiliac joint or postcervical vertebrae involvement (13). The lack of any similar paleopathological or written evidence to support a history of RA in Europe pre-1800, has led to the suggestion that the disease may have been transmitted from the so-called New World to the Old (14). The first detailed description of RA in Europe has been attributed to Augustin-Jacob Landré-

Beauvois who described in 1800, an inflammatory polyarthritis he termed 'primary aesthenic gout'. The term rheumatoid arthritis was first proposed in 1859 by Sir Alfred Garrod, but was not adopted until 1921 by the British Ministry of Health, 1941 by the American Rheumatism Association (ARA) and 1957 by the International League Against Rheumatism (ILAR) (14, 15).

Paleopathological evidence indicates that PsA, like osteoarthritis (OA), AkS and gout, has existed since antiquity (16). Skeletal remains uncovered from a fifth century monastery in the Judaen desert displayed typical features of PsA including characteristic arthritis mutilans, and their presence in a monastery suggests that psoriasis was, at this time, considered biblical leprosy (17). The discovery of a 13<sup>th</sup> century male skeleton in England exhibiting typical PsA characteristics such as widespread arthritis with ankylosis of the interphalangeal joints and 'cup and pencil' deformity of the distal interphalangeal joints, further confirms its history (18). It is perhaps surprising then that unlike RA, PsA was not accepted as a disease in its own right until relatively recently when in 1964 it was included in the ARA classification of arthritis (11) following substantial support from epidemiological and clinical studies (19-22).

### **1.1.2 Definition and Classification**

RA is commonly defined as a chronic inflammatory disease of the synovium causing joint damage and destruction of bone resulting in disability and increased mortality. The current accepted criteria for the classification of RA have been set by the 1987 revised American College of Rheumatology (ACR) criteria. The criteria are: (1) morning stiffness, (2) simultaneous arthritis in 3 or more joint areas with soft tissue swelling or fluid, (3) arthritic swelling of the hand joints, (4) symmetrical presentation of arthritis, (5) rheumatoid nodules over bony prominences, extensor surfaces or subarticular regions, (6) serum rheumatoid factor (RF) positive and (7) radiographic changes including erosions or bony decalcifications. For classification purposes, a patient is diagnosed as having RA if they present with at least 4 of the 7 criteria, with the first four criteria present for at least 6 weeks (23).

In contrast to RA, criteria for the classification of PsA have not been definitively established. Initially delineated from RA based on composite clinical features including



asymmetrical erosive polyarthritis, particularly with distal interphalangeal involvement, association with psoriasis and the absence of serum RF (24), between 3 and 5 distinct clinical sub-groups of PsA have since been proposed (25-27). However, as patterns of clinical involvement can over-lap between subgroups there remains continued debate as to the most appropriate diagnostic criteria for PsA. One example is a classification system proposed by Veale et al comprising (1) asymmetric oligoarthritis, (2) frequently erosive, seronegative, symmetric polyarthritis and (3) sacroiliitis with predominant spondylitis (28). Simpler classifications based upon synovial inflammation or enthesitis have also been proposed (29, 30). The difficulty in defining specific criteria is further confounded by the evolving nature of joint involvement. Over 60% of patients change from their initial pattern, for example an oligoarticular pattern may evolve to polyarthritis and vice-versa (31, 32). Importantly it has been recognised that PsA is not the mild disease originally believed but can, like RA, cause progressive functional disability (33) and increased mortality (34).

### **1.1.3 Epidemiology**

Community and population based studies of the incidence and prevalence of RA have been numerous and indicate considerable geographic variation in disease occurrence. In the United Kingdom (UK) the prevalence of RA in the adult population is estimated at 0.8% (5) with an annual incidence of 36 per 100,000 in women and 14 per 100,000 in men (35). In other Northern European areas and in North America the prevalence is estimated at 0.5-1.1% of the general population with an annual incidence of 20-50 per 100,000 inhabitants (36-39). An even higher prevalence of 5.3-6.0% and incidence of up to 90 in 100,000 has been reported in Native Americans and Native Alaskans, potentially linked to high human leukocyte antigen (HLA) DRB1\*1402 genetic risk associations (discussed in *1.1.6.1.2*) (40). Prevalence is lower in Southern European areas at 0.3-0.7% and annual incidence of 10-20 per 100,000 inhabitants (41-43). Studies from developing countries have been few but report similarly low prevalence between 0.1% and 0.3% (44).

Rather fewer epidemiological analyses have been undertaken on PsA. Prevalence is estimated at between 0.2-0.5% in Northern European and North American Caucasians (45, 46). It is less prevalent in African-Americans, Native Americans (unlike RA) and South American Indians (47, 48). Prevalence of PsA is increased in patients with psoriasis,

occurring in 6-42% of sufferers (7-10). Only a small number of population based incidence studies of any note have been undertaken on PsA. Incidence has been reported as between 6-8 in 100,000 in Finland (49, 50), 8 in 100,000 in Sweden (51), 6.62 in 100,000 in USA (52), 3.02 in 100,000 in Northwest Greece (53) and 3.5 in 100,000 in the UK (54). Importantly, the last was a study of early PsA and may represent an underestimate of prevalence, particularly as psoriasis does not precede arthritis in 15-25% of cases (55). Until common standardised criteria are established for the diagnosis of PsA it remains difficult to determine its true incidence in different populations.

Both RA and PsA can occur at any age, however RA most commonly occurs between the ages of 40 and 70 (56) whilst PsA usually develops between 35 and 45 years of age (57). Of interest, while RA favours females over males estimated at 2.5:1 and PsA occurs with equal frequency in both males and females, in both diseases an earlier age of onset and the female gender are associated with disease severity (58) (59).

#### **1.1.4 Clinical Features**

RA and PsA are inflammatory conditions. PsA and RA patients present a heterogeneous and sometimes overlapping combination of articular and extra-articular features, as summarised in Table 1.1. Although PsA often displays many features in common with the spondyloarthropathies such as enthesopathy and HLA-B27 associations, some clinical features overlap with RA, particularly in severe arthritis. For example, although PsA is classically described as presenting a predominantly asymmetric distribution, symmetry is demonstrated by up to 53% of patients who go on to develop polyarticular disease (60). Moreover, it has been proposed that asymmetry is not a distinctive qualitative feature of PsA, but a function of low clinical joint involvement particularly in early disease, and as such is not a robust diagnostic criterion (61). In addition psoriasis, the defining extra-articular feature of PsA, does not develop in all patients. Therefore PsA is not solely distinguished from RA via the clinical presentation, but also by the lack of gender preference (62), less aggressive (usually) clinical course (58, 63) and absence (usually) of rheumatoid factor (20, 64).

**Table 1.1** Comparison of clinical features of RA and PsA

	RA	PsA
Distribution	Symmetrical (65)	Asymmetrical(25)
Articular Features	Palpation tenderness, synovial thickening, effusion, erythema, decreased range of motion (23), ankylosis, subluxation (66, 67).	Dactylitis (30%) (68-70); Enthesopathy(71); Spondyloarthropathy (33%) (sacroiliitis, spinal disease)(60); Distal interphalangeal joint involvement(72).
Extra-articular features	Skin (rheumatoid nodules, vasculitis) (73, 74) ; Ocular (keratoconjunctivitis sicca, iritis, episcleritis) (75, 76); Oral (salivary inflammation)(77); Respiratory (78); Cardiac (79, 80); Neurological (81); Hepatic (82); Haematological (anaemia, thrombocytosis) (83); Vascular (vasculitis) (84); Ischaemic heart disease (IHD) (85, 86).	Skin: Psoriasis (psoriasis vulgaris (predom.), pustular psoriasis, guttate psoriasis, erythroderma), Nail lesions (67-90%) (pitting, ridging, onycholysis); Ocular (7-33%) (Conjunctivitis, iritis)(25, 76); Aortic incompetence (<4%) (25, 28)
Rheumatoid Factor	Positive (80%) (23, 87)	Negative (90%) (25, 60, 88)
Standard Mortality Ratio	2.3-2.7 (89, 90)	1.59-1.65 (91)

## 1.1.5 Histopathology

### 1.1.5.1 The Normal Synovium

The normal synovium covers the inner surface of joints and is a relatively acellular structure of approximately 0.5-5mm thickness, with a delicate intimal lining layer of approximately 1-2 cells in depth and a thicker sublining connective tissue layer. The intimal lining layer is composed of two types of interdigitating synoviocytes, the cluster of differentiation (CD) 68<sup>+</sup>/major histocompatibility complex (MHC) II<sup>+</sup>/FcγR<sup>+</sup>/CD14<sup>+</sup>/CD45<sup>+</sup>/α6β1-integrin<sup>-</sup> macrophage-like synoviocyte (or 'type A') and the vascular cell adhesion molecule-1 (VCAM-1)<sup>+</sup>/CD55<sup>+</sup>/α6β1-integrin<sup>+</sup>/uridine diphosphoglucose dehydrogenase (UDPGD)<sup>+</sup> fibroblast-like synoviocyte (FLS, or 'type B') (92, 93). The sublining layer forms a matrix composed of collagen types I, III, IV, V and VI as well as fibronectin, lamin and proteoglycan, interspersed with macrophages, adipocytes and VCAM-1<sup>+</sup>/CD55<sup>+</sup>/α6β1-integrin<sup>+</sup>/UDPGD<sup>-</sup> fibroblasts (94). The synovium maintains homeostasis and viscosity of the synovial fluid, which fills the joint cavity, through production of hyaluronidan, lubricin and surface-active phospholipid, synthesised by both type A and type B cells (95-97). The normal synovial fluid is also a relatively acellular compartment with mean counts of 35cells/μl (98). In combination, the normal synovium and synovial fluid provide joint protection and lubrication as well as a means by which nutrients can be transported to the joint cartilage and debris can be cleared (99).

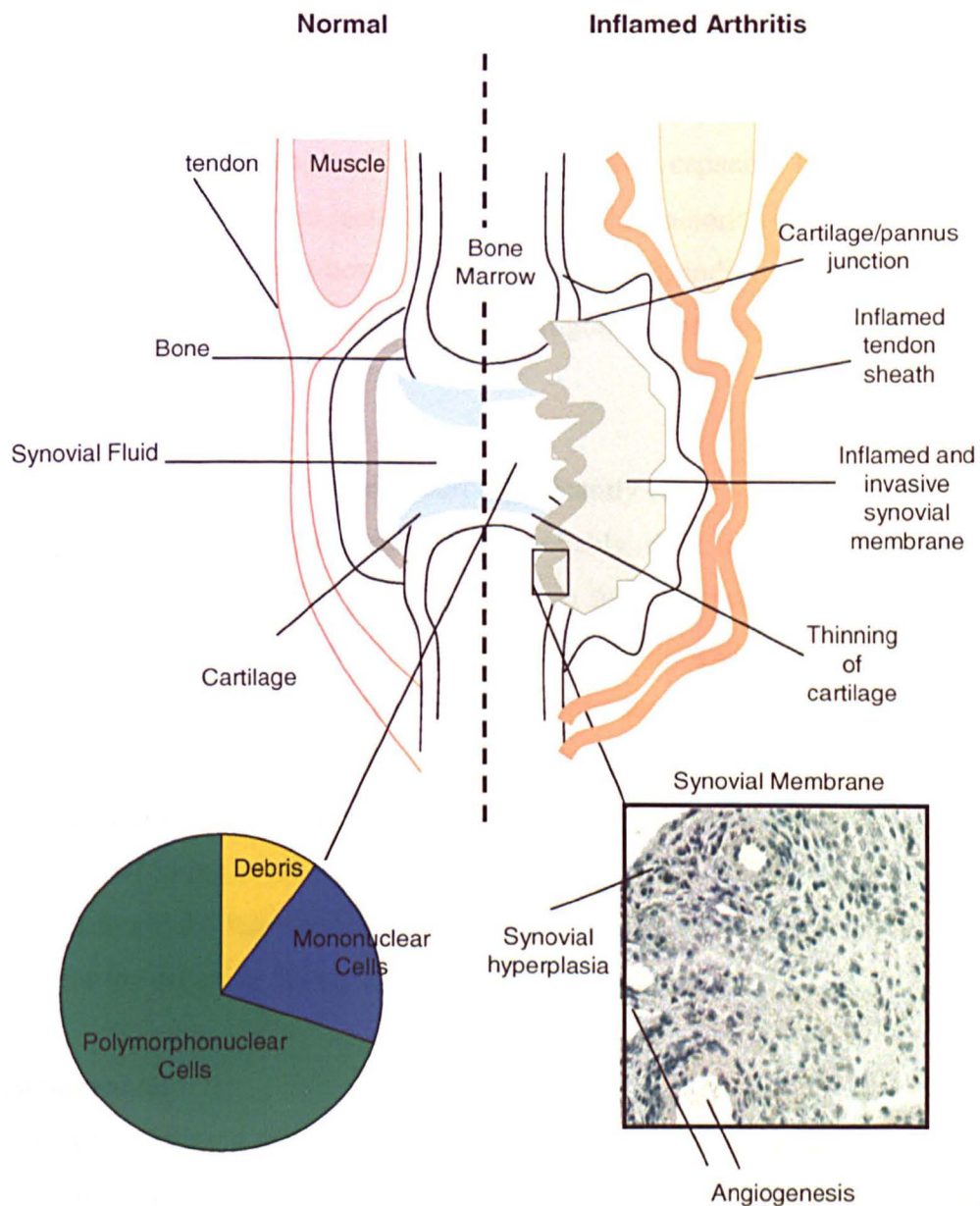
### 1.1.5.2 The Inflamed Synovium

During chronic arthritic inflammation, the synovium rapidly evolves from a protective nutritional structure into an aggressively invasive tissue, characterised by hyperplasia of the intimal lining layer, pronounced angiogenesis and concomitant influx of immune cells to the sublining layer (Figure 1.1). The resulting inflammatory activated synovial tissue, also termed "pannus", causes irreversible destruction of cartilage and bone through osteoclast-mediated bone resorption. Macrophages and synovial fibroblasts release matrix-metalloproteinases (MMPs), aggrecanases and serine proteases that digest collagen and proteoglycans, degrading the extracellular synovial matrix. In addition, the synovial fluid expands abnormally, accumulating in the order of 10-1000 cells/μl (99), enriched in predominantly polymorphonuclear cells, but also mononuclear cells including monocytes, myeloid dendritic cells (mDC), plasmacytoid (p) DC, T cells, B cells and natural killer

**Figure 1.1 Schematic representation of a normal joint compared to an inflamed arthritic joint, showing the associations between the joint capsule, synovium, bone and cartilage\***

In the normal joint the relatively thin, acellular synovium maintains homeostasis and viscosity of the synovial fluid, which fills the joint cavity. Together the synovium and synovial fluid provide joint protection and lubrication as well as a means by which nutrients can be transported to the joint cartilage and debris can be cleared. In the inflamed arthritic joint the synovium (or “pannus”) thickens, undergoing hyperplasia, angiogenesis and leukocyte infiltration, invading neighbouring cartilage and bone through osteoclastogenesis. The synovial fluid also expands, accumulating polymorphonuclear cells, mononuclear cells, cellular and matrix debris. (Original magnification x400).

\*Adapted from (101)



(NK) cells as well as cellular and matrix debris (101-106). Although the fundamental features of synovial inflammation are similar in RA and PsA, there are a number of qualitative and quantitative differences between the two diseases.

#### ***1.1.5.2.1 Synovial thickening***

In RA, the intimal lining layer thickens to a depth of over 8 cells, whereas in PsA intimal lining layer thickening is in the order of 2-3 cells deep (107, 108). Macrophage-like synoviocytes account for up to 80% of the hyperplastic intimal lining layer however only a small proportion of this is thought to be attributable to *in situ* proliferation. Reduced Ki67 staining (109) and synovial explant culture proliferative capacity (110), as well as expression of leukocyte common antigen (LCA) on the majority of cells in the RA synovial lining demonstrates their bone marrow origin (111), indicating that the majority of synovial thickening can be accounted for by immigrating monocytes and macrophages from the peripheral blood.

Consistent with this a number of studies report significantly fewer macrophages in the PsA synovium compared to RA synovium, most probably due to lower expression of macrophage recruiting selectin CD62E (E-selectin) in the former compared to the latter, hence the thinner intimal lining layer (107, 112). However, the validity of this observation has been disputed as comparative studies may not have matched RA and PsA patients for duration of disease, hence the differences may be artefact (113). In addition, cadherin-11 has recently been described at elevated levels in the rheumatoid synovial lining layer and is a key determinant of lining layer formation by facilitating FLS-FLS adhesion (114). The expression of cadherin-11 has not yet been described in PsA, but an altered expression could account for the differing lining layer thickness between RA and PsA.

#### ***1.1.5.2.2 Angiogenesis***

Angiogenesis is a prominent early feature of synovitis, fundamental to disease pathology by transmitting oxygen and nutrients to the hypertrophic synovium in addition to providing a means by which inflammatory cells are recruited to the anatomical compartment. In RA and PsA the pro-angiogenic factors angiopoietin-1 and -2, vascular endothelial growth factor (VEGF) and transforming growth factor  $\beta$  (TGF $\beta$ ) are detectable at early stages of disease. However, levels are significantly higher in PsA as compared to RA, and this can

be correlated to significantly more blood vessels per square millimetre in the former compared to the latter (107, 115). Moreover, and in common with other spondyloarthropathies, the PsA synovium contains a tortuous vasculature, whereas the RA synovium contains a straight vasculature in 89% of early cases, and this specific difference may be of diagnostic value early in disease (116, 117). In addition, pro-angiogenic factors including insulin-like growth factor (IGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (KGF) and platelet derived growth factor (PDGF), produced by macrophages and synovial fibroblasts, are up-regulated in synovial fluid and have been co-localised to perivascular regions of the sublining layer of the synovium (118, 119). Of importance, angiogenic inhibitors including endostatin and thrombospondin-1 and -2 have also been identified in RA, suggesting that the marked angiogenesis results from an imbalance that favours angiogenic promoters over inhibitors (120-122).

#### ***1.1.5.2.3 Cellular Immunopathology***

A characteristic of the inflamed synovium in RA and PsA is the rapid influx of mononuclear cells including CD4<sup>+</sup> and CD8<sup>+</sup> T cells, plasma cells, B cells, NK cells, monocytes, mast cells and dendritic cells (DC) (101-105, 107, 123-125). The cellular infiltrate is in a predominantly perivascular distribution and recruitment is enabled by enhanced expression of adhesion molecules including CD62E (E-selectin) on endothelial cells, intracellular adhesion molecule (ICAM) -1 and VCAM-1 (126). Fibroblast-like synoviocytes (FLS) and macrophages (M $\phi$ ) within the inflamed synovium have been demonstrated to be comparable between RA and PsA (127). In addition, CD4<sup>+</sup> T cells are the most abundant inflammatory cell in the synovium, however their presence in the PsA synovium is significantly less than that observed in RA SM (107, 127, 128). In contrast, CD8<sup>+</sup> T cells exceed CD4<sup>+</sup> T cells in the PsA, but not RA, SF and enthesitis. Furthermore CD8<sup>+</sup> T cells infiltrate lesional psoriatic plaques earlier than do their CD4<sup>+</sup> T cell counterparts (129, 130). This may suggest a role for CD8<sup>+</sup> T cells in PsA pathology, commensurate with MHC class I genetic associations to PsA (discussed in *1.1.6.1.2*) and CD8<sup>+</sup> T cell receptor (TCR) oligoclonality (discussed in *1.1.7.2.1*)(131).

The organisation of HLA-DR expressing CD4<sup>+</sup> T cells into lymphocytic aggregates in close association with MHC expressing macrophages and DC, as well as B cells, has led to



the frequent referral to the inflamed synovium as an ectopic lymphoid organ. However, the appearance of lymphoid-like architecture has been identified in only 50% of PsA cases, with the majority expressing a diffuse lymphocytic infiltrate. Similarly, in RA lymphocytic aggregates occur in only 40-50% of cases, with germinal centre formation occurring in as few as 23% of cases (132). Moreover, immunohistochemical analysis of 11 RA synovial biopsies by Page and Miossec indicated a fundamental lack of characteristic lymphoid features, including germinal centre formation in only 3/11 samples, as compared with paired lymph node biopsies (133). The extent to which lymphoid-like features correlate to disease duration in PsA has not been addressed, however studies by Baeten et al. and Tak et al. suggest that there are no significant histological differences between early and established RA, although significant differences can be observed between clinically active and inactive disease (116, 134). Moreover, the lack of classical lymphoid topographical segregation in the majority of cases may enhance disease pathology, particularly with respect to the potential for *in situ* antigen presentation.

#### **1.1.5.2.4 Joint erosion**

The release of proteolytic enzymes by macrophages and synovial fibroblasts is considered a key early event in the pannus mediated degradation of cartilage and bone intrusion. MMP-1 (collagenase) expression in synovial fluid, serum and synovium derived mRNA correlates to the development of radiological erosions in early, and established, RA and PsA (135-137). MMP-3 (stromelysin) is also elevated in PsA and RA serum and synovial fluid (137-139). It has been proposed that the low level resistance of collagen VI to MMP mediated degradation may account for the continued stability of the synovial matrix during active inflammation (140), however collagen type II is the dominant component of cartilage.

Activation of bone-resorbing osteoclasts by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor  $\kappa$ B ligand (RANK-L), both of which are up-regulated by IL-1 and TNF- $\alpha$ , is a critical event in bone damage in both RA and PsA (141-143). In PsA, bony erosions, particularly at the pannus/cartilage interface, are less common and appear at a slower rate than in RA, and instead present in a distal pattern most probably at the enthesal/bone junction (26, 144). Consistent with this, levels of aggrecans, components of the cartilage structure and an indicator of cartilage destruction,

are lower in PsA synovial fluid than in RA synovial fluid (145, 146). Recent analyses by Jimenez-Boj (147) and Bugatti (148) revealed that in erosive inflammatory arthritis, bone marrow exposed by violation of the cortical barrier by the adjacent inflamed synovial tissue, may constitute an additional disease compartment as a pool from which mature B cell aggregates are formed. Of interest, it has been hypothesised that these B cell aggregates form a physical barrier shielding the bone sub-cortical bone marrow from the invading pannus, in addition to triggering new bone formation (147).

### **1.1.6 Aetiology**

The precise aetiology of RA and PsA is unknown, but is believed to comprise a composite blend of numerous genetic, environmental, medical, lifestyle, hormonal and reproductive risk factors as well as dysregulated immunological processes.

#### **1.1.6.1 Genetic Factors**

There is strong evidence from population and twin studies to support the influence of heritable factors on the disease development of RA and, to a lesser extent, PsA as the former has been the focus of larger and more numerous analyses than the latter. Studies suggest that as much as 60% of a population's pre-disposition to RA can be accounted for by genetic factors (149). In both RA and PsA, association studies with human leukocyte antigens (HLA), genome-wide linkage scans and analysis of the major histocompatibility complex (MHC) region, have revealed that the genetics of both diseases are complex and unique, with polygenic characteristics of inheritance influencing occurrence, expression and age of onset (150).

##### **1.1.6.1.1 Twin Studies**

Twin studies have indicated that in RA, there is higher concordance among monozygotic twins at 12-15% than in dizygotic twins at 4% (151, 152). No formal twin studies have as yet been undertaken on PsA, probably due to its low frequency. Of interest, one case has been reported by Moll and Wright concerning triplets, two identical and one non-identical, where the identical twins developed psoriasis with arthritic associations but the non-identical twin developed neither (153). Furthermore, studies of psoriasis alone have revealed a strong concordance among monozygotic twins at 65-72% compared to dizygotic twins at 15-30% (154-156). In addition, several studies have identified familial clustering

in PsA with a first-degree relative risk of 8.3-14% (153, 157), thus supporting heritable risk in PsA aetiology.

#### ***1.1.6.1.2 HLA Associations***

RA and PsA have strong associations with HLA genes, which reside in the MHC region, and in both cases these have been found to be predictive of progression of clinical damage. In RA it is estimated that 50% of the genetic component of disease susceptibility may be accounted for by genes in the HLA region, particularly HLA-DRB1 (149, 158). HLA associations in RA have been identified as HLA-DR1 alleles (\*0101, \*0102), the HLA-DR4 alleles (\*0401, \*0404, 0405, 0408), the HLA-DR6 alleles (\*1401 and \*1402) and HLA-DR10 alleles (\*1001)(159-162). The susceptibility across different ethnic populations to RA is closely correlated to the expression of the short amino acid sequence QKRAA/QRRAA in the third hypervariable region common to all HLA-DRB1 alleles associated with RA, and this is commonly defined as the “shared epitope” hypothesis (160, 163).

Unlike RA, PsA is not strongly associated with the HLA class II alleles, but is associated with the HLA class I alleles. Reported associations include the HLA antigens A3, Cw6, B7, B13, B17, B27, B38, B39 DR7 $\alpha$ , DRB010 and HLA-DRB0301(164-168). HLA-B27 is one marker considered indicative of the association of PsA with the spondyloarthropathies and may also be predictive of clinical features such as joint deformity (169). Other HLA antigens considered predictive of clinical progression include HLA-DR7 and HLA-B39, which are associated with joint damage when in the presence of HLA-B27, whereas HLA-B22 is protective (91). Individuals exhibiting severe PsA on a par with RA have increased levels of HLA-DR4 (166, 170). PsA has also been associated with polymorphisms in the MHC class I chain-related gene A (MICA), which was not associated with psoriasis alone (171, 172). MICA is expressed by heat-stressed epithelial cells and is recognised by CD8  $\alpha\beta$  T cells,  $\gamma\delta$  T cells and NK cells, and thus polymorphisms in this loci may have adverse consequences for the innate immune response (173), a position supported by studies highlighting the integral association of CD8 T cells and NK cells with PsA immunopathology.

While the association of the HLA-DR alleles with RA have been known for at least 27 years, recent analyses have indicated a strong association between the shared epitope containing HLA-DRB1\*0404 and HLA-DRB1\*0401 and autoantibodies against cyclic citrullinated peptides (anti-CCP antibodies) (discussed in 1.1.7.2.3) (174-177). It has been proposed that the HLA-DRB1/anti-CCP antibodies association represents a specific disease phenotype (177), a proposition supported by Verpoort et al. who showed that HLA-DR3 is not associated with anti-CCP positive arthritis, but is associated with anti-CCP negative arthritis (178). Similar analyses in PsA have been limited, but one analysing 126 PsA patients showed that while only 5.6% of patients were positive for anti-CCP antibodies, of these patients there was a significant association with the HLA-DRB1 shared epitope and disease progression (179).

A number of theories have been proposed to account for the strong association of HLA-DR alleles and inflammatory arthritis, including multiple copies, disease penetrance, time of onset and severity. The shared epitope may act as a receptor for arthritogenic peptide, directing the T cell repertoire towards an autoreactive response. The demonstration of follicular aggregates containing lymphocytes and high HLA-DR expressing antigen-presenting cells (APC) within the inflamed synovia of patients with active RA is consistent with such a hypothesis (180). There may also exist molecular mimicry of the shared epitope by pathogens such as Epstein-Barr virus (EBV), Cytomegalovirus (CMV) and DNA J heat shock protein from E.coli (discussed section 1.1.7.1), thus serving as a target for autoreactive T cells (158, 181). The effect may be exacerbated by the unique contacts between T cell receptors and MHC molecules on APC in follicular aggregates within the inflamed synovia leading to enhanced T cell reactivity (15, 180).

#### ***1.1.6.1.3 Non-HLA genetic associations***

Genome wide linkage scans have identified a number of non-HLA genes that may explain the remainder of the genetic component of susceptibility to RA and PsA. Regions have been located on chromosome 1q, 5p, 3q13, 6q, 8p, 14q and 16p (182-185).

***1.1.6.1.3.1 Protein tyrosine phosphatase N22*** A number of genes emerging as convincing candidates for RA associations include that encoding protein tyrosine phosphatase N22 (PTPN22), which is linked to negative regulation of T cell activity (186, 187). A recent study from Sweden found that in a patient cohort, the combination of polymorphisms in

the PTPN22 gene 1858 and positive anti-CCP antibody titres conferred 100% specificity for RA development (188). However a similar German study of 390 patients found that PTPN22 1858 was associated with RA irrespective of RF or anti-CCP production (189). Of interest, synergy between the SNP in the minor PTPN22 allele *R620W* and the shared epitope have been identified in the early failure of self-tolerance toward the cartilage determinant C1 (III) in RA (190). Only moderate associations between PsA and PTPN22 polymorphisms have been observed in a patient cohort in Canada (191), however other studies have detected no associations (187) and more analyses are required to clarify the strength, if any, of this association.

**1.1.6.1.3.2 Macrophage inhibitory factor** Polymorphisms in genes encoding macrophage migration inhibitory factor (MIF) (192, 193), which has pro-inflammatory properties, have also been associated with RA. Although no link with MIF has yet been observed in PsA, MIF has been positively associated with disease in a cohort of psoriasis patients (194).

**1.1.6.1.3.3 Programmed death receptor 1** A number of studies have identified SNP in the gene encoding programmed death receptor 1 (PDCD-1), an immunoinhibitory receptor expressed by T and B cells, which confers susceptibility to RA (195, 196). A relationship between PDCD-1 polymorphisms and disease susceptibility has also been identified in SLE (197) and diabetes mellitus but not PsA (198).

**1.1.6.1.3.4 Peptidylarginine deiminase type 4** A relationship of SNP in peptidylarginine deiminase type 4 (PADI4), a gene that encodes enzymes to change arginine into citrullinated proteins, has been associated with RA but not PsA (199, 200). PADI4 expression has been identified in macrophages and granulocytes within rheumatoid synovium.

**1.1.6.1.3.5 Caspase recruitment domain-containing protein-15 (CARD15)** Genome wide scans of PsA have been few, but a susceptibility locus has been identified at chromosome 16q (201), which overlaps with CARD15, a susceptibility gene for Crohn's disease (202). Interestingly, prevalence of psoriasis and PsA in Crohn's patients exceeds that in the general population (203, 204). CARD15 mutations may potentially inhibit the innate immune response to pathogens by altering recognition of bacterial polysaccharide and subsequently diminishing nuclear factor (NF)  $\kappa$ B activation (205).

Strong support for the vital role of genetic risk factors in arthritic aetiology has come from numerous animal models particularly those involving genetic-risk DBA-1. However, a number of models have been described whereby collagen-induced arthritis (CIA) can be induced in non-genetically sensitive animals provided that a sufficiently strong adjuvant is given (206). This evidence, coupled with numerous models describing infection induced arthritis, supports a definite role for environmental factors in disease aetiology.

#### **1.1.6.2 Environmental and Medical Factors**

Environmental factors, including infectious agents and trauma, have long been suspected as potential triggers in the pathogenesis of RA and PsA.

##### ***1.1.6.2.1 Infectious agents***

The putative association of RA and PsA with chromosomal mutations that may diminish or dysregulate innate immune responses is consistent with a contribution of pathogens in disease risk. Indeed, psoriatic flares have been closely linked to streptococcal infection (207-209). Moreover keratinocytes within psoriatic plaques produce large quantities of antimicrobial peptides  $\beta$ -defensins and cathelicidins commensurate with an inflammatory response to pathogens (210).

Several infectious agents including bacteria, mycobacteria, viruses and parasites, have been implicated in the aetiology of RA. Mycoplasma such as *M. fermentans* (211-214) and *M. pneumoniae* (215), have been implicated as cofactors in inflammatory arthritis, particularly RA. Proposed viral triggers associated with RA include EBV, CMV, rubella virus and parvovirus B19 (216-219). Comparative analysis of existing data has demonstrated synovial virus infections, in particular coincident infections, are far more common in autoimmune arthritis, such as RA and PsA, than in reactive arthritis or septic arthritis (220, 221). Associations between infectious agents and RA remain controversial as serological studies vary widely between patient cohorts and many have been negative when applied to large cohorts of recent onset disease. Combined with the lack of time and space clustering in RA onset, which would be expected in the case of a strong and direct association between disease and specific infection, and the link becomes somewhat tenuous (222). Of interest however is the relatively recent increase in the incidence of PsA in Africa, which has been attributed to infection with the human immunodeficiency virus

(HIV) (223, 224). This has been accompanied by the contrary observation that HIV infection appears to be associated with RA remission. It is possible that HIV triggered T cell dysregulation or regulatory T cell depletion may contribute to, or indeed cause, PsA, supported by the lack of an HLA-B27 antigen association in PsA in sub-Saharan African HIV patients (225). Importantly, the fact that RA and PsA are not only clinically distinct, but immunologically and pathologically distinct entities is highlighted.

#### ***1.1.6.2.2 Blood Transfusion***

Studies have noted a consistent association between previous blood transfusion and subsequent RA development, possibly due to transfusion induced RF production (226-228). The risk however appears to have dropped in recent years coincident with new blood processing techniques.

#### ***1.1.6.2.3 Trauma***

In RA there appears to be little evidence to support a link between physical trauma and disease onset. In PsA trauma likened to Koebner's phenomenon, whereby psoriasis occurs following mechanical, physical or chemical irritation of the skin, is recognised as an important aetiological factor in disease onset (229-231). It is thought that arthritis may be triggered by trauma due to the release of vasoactive, pro-inflammatory neuropeptides such as substance P (232, 233). This hypothesis is supported by case studies identifying the inhibition of arthritis of the distal interphalangeal joint by traumatic digital paralysis (234, 235). Furthermore substance P release from the synovial membrane (SM) to the synovial fluid (SF) can be blocked by nerve damage (234). Additional credence to the link between PsA and physical trauma is the established observation that PsA, but not RA, is associated with enthesopathy, an inflammatory involvement of the tendon, ligament and joint capsule attachments collectively known as the enthesis. Indeed fibrocartilage, a prominent feature of the enthesis organ, increases with joint stress, a significant event in genetically susceptible individuals as fibrocartilage directed autoimmunity has been linked to both the human spondyloarthropathies and animal models of disease (236-238). Moreover enthesopathy has been found to feature lymphocyte, plasma cell and polymorphonuclear cell infiltration (239), as well as bone structure alterations (erosion and proliferation), linked to adjacent synovitis and possible fibrocartilage autoimmunity (240-242). Angiogenesis is also highly active in early onset PsA (more so than in RA), resulting in a

pronounced tortuous vessel pattern in up to 93% of PsA patients but not in RA patients (243), and this is consistent with trauma as a risk factor for PsA (116).

### **1.1.6.3 Lifestyle Factors**

To date, there has been a paucity of studies analysing the association of various lifestyle factors and the relative risk of developing PsA. There have however been a number of studies examining the influence of lifestyle factors on the occurrence of RA.

#### ***1.1.6.3.1 Smoking***

Smoking significantly increases the risk, severity and outcome of RA in men and women, particularly in heavy smokers, supported by both cross-sectional and longitudinal epidemiological analyses (244-246). While the exact mechanism of association is unclear, smoking has been linked to increased RF production, decreased antioxidants in the blood and altered hormone levels (247). A significantly increased risk of developing seropositive RA has also been observed in shared epitope HLA-DR positive smokers (248). Of particular interest, Klareskog et al. have extended this observation by identifying a specific relationship between tobacco smoke induced peptide deimination and subsequent anti-CCP antibody production in HLA-DRB1 positive smokers, thus providing evidence of a biological explanation for smoking as a risk factor (249).

#### ***1.1.6.3.2 Socioeconomic Status***

Increased socioeconomic status has been shown by a number of studies to be inversely correlated with course, outcome and risk of RA (250-252), although the relative risk appears more robust in the female population (252).

#### ***1.1.6.3.3 Obesity and Diet***

Obesity has been found to be a risk factor for RA in women, but not men (228, 253). Dietary factors contributing to risk of RA include high red meat and meat product consumption (254), while diets high in olive oil, cooked vegetables, alcohol consumption and omega-3 long chain polyunsaturated fatty acid (PUFA) found in oily fish, may have a protective effect (253, 255, 256). Considering the epidemiological evidence indicating low prevalence and incidence of RA in Mediterranean regions (see section 1.1.3), it appears that a Mediterranean style diet may be protective against the onset of RA.



#### 1.1.6.4 Hormonal and Reproductive Factors

RA is more common in women than men, particularly in the pre-menopausal age group, suggestive of a role for hormonal factors in disease susceptibility. Of interest, pregnancy is associated not only with remission of existing RA, but reduced susceptibility to RA, followed by an increased susceptibility in the post-partem period (257, 258). However, the increased susceptibility of females to RA is only true of individuals with low HLA-DRB1 shared epitope expression. The gender bias decreases to absentia with increasing numbers of shared epitope alleles (259). Furthermore, a study linking PTPN22 1858 polymorphisms with the HLA-DRB1 shared epitope in a male, but not female, cohort may indicate that the genetic contribution to disease is more prominent in men (189).

PsA shows no gender bias, although a nested case control study of the Rochester Epidemiology Project reported pregnancy as a protective factor in the risk of developing PsA in patients with pre-existing psoriasis (260). The same study indicated that corticosteroid use in psoriasis increased the risk of developing PsA. The oral contraceptive pill has been recognised since 1968 as reducing the relative risk, and potentially the severity, of RA, but similar studies have not yet been undertaken in PsA (261, 262).

The considerable and interrelated contribution of both genetic and environmental factors to disease aetiology, as eloquently demonstrated by the strong association of cigarette smoking, HLA-DR shared epitope and anti-CCP antibodies to disease susceptibility, infers involvement of both the innate and adaptive immune system to the underlying immunopathology of disease. This is supported by a murine model whereby collagen-induced arthritis can be demonstrated in non-genetically susceptible C57BL/6 (H-2b) mice provided that the adjuvant gives a sufficiently strong environmental insult (206). In addition spontaneous arthritis has been reported in numerous genetically susceptible models such as the MRL *lpr/lpr* model of arthritis (263, 264). Models combining both of arms of the immune response (innate and adaptive) induce severe, chronic arthritic disease, as exemplified by collagen type II challenge in oil-induced arthritis susceptible rats (265).

### **1.1.7 Immunopathology**

RA and PsA are generally acknowledged to be diseases of dysregulated inflammation, driven and maintained by multiple interacting components of the innate and adaptive immune system.

#### **1.1.7.1 Innate Immunity**

The innate immune response constitutes the body's first line of defence against infectious organisms and is composed of constitutive and inducible mechanisms. The constitutive mechanisms are continuously expressed and include, for example, physical barriers provided by mucosal layers and epithelial cell shedding at surfaces continuously exposed to microorganisms including the skin, intestinal and genital tracts. In contrast, the inducible mechanisms of innate immunity are induced by DC, macrophages, NK cells, T  $\gamma\delta$  lymphocytes and neutrophils following recognition of pathogen-associated molecular patterns (PAMPs) expressed by the majority of microorganisms. PAMP recognition is mediated by pattern-recognition receptors (PRR), including lectins, mannose-binding receptors, dectin-1, DEC-205, pentraxins, NOD proteins and toll-like receptors (TLR) expressed, to a varying degree, by the cells of the innate immune system. PRR signalling triggers diverse functions such as complement activation and opsonization; phagocytosis and clearance of pathogens and cellular debris; and antimicrobial peptide, superoxide, nitric oxide, pro-inflammatory cytokine and chemokine release. While bacteria, mycobacteria, viruses and parasites have long been implicated in triggering and exacerbating RA and PsA (211-214, 220, 221), the relatively recent identification of active TLR ligands within the inflamed synovium has renewed interest in the role of innate immunity in disease pathology (see also 1.2.2.5). In particular TLR2, and TLR4, and their endogenous ligands, have been identified in the inflamed synovial compartment (266-269). Moreover they have been demonstrated to be central to a number of murine models of arthritis including that induced by collagen type II and LPS (270). Of importance, triggering of these TLR contributes significantly to matrix metalloproteinase (MMP) production, as well as the release of cytokines and chemokines and co-stimulatory molecule up-regulation by DC that could dictate the skewing of ongoing adaptive immune response.

It is likely that exogenous or endogenous TLR agonists expressed within the synovial compartment may promote the perpetuation of chronic inflammation, and potentially even provoke the leukocyte infiltration to the synovium. However, the presence of immunocompetent cells in the inflamed SM coupled with the identification of autoantigens in patient sera and SF underlines the contribution of the adaptive immune response to chronic inflammation (Fig. 1.2).

### **1.1.7.2 Adaptive immunity**

In contrast to the innate immune response, adaptive immunity revolves around cellular and humoral immunity induced by clonally expanded antigen specific  $\alpha\beta$  T cells and B cells and forms the basis of immunologic memory.

#### **1.1.7.2.1 T cells**

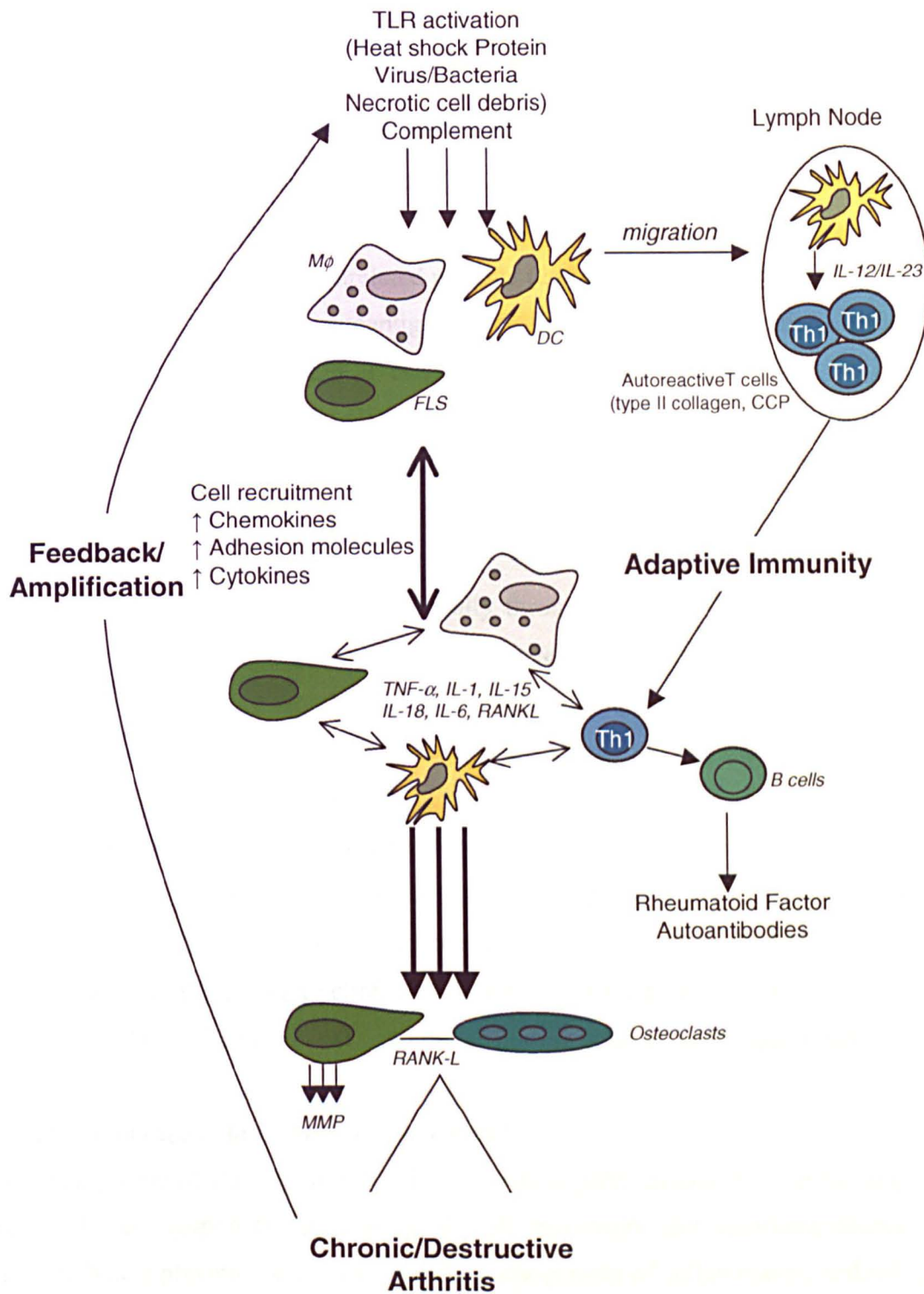
$\alpha\beta$  T cells can be divided into two subgroups,  $CD4^+$  and  $CD8^+$ .  $CD4^+$  T cells, also referred to as T helper (Th) cells, confer cellular immunity (Th1) or humoral immunity (Th2) (271). Strong evidence indicates a role for  $CD4^+$  T cells in the pathogenesis of both RA and PsA. First,  $CD4^+$  T cells are enriched to between 16-75% of all infiltrating cells in RA (272) and PsA (107, 128) SF and SM. Second, the strong association of HLA genes to aggressive pathology in RA and PsA, may reflect presentation of arthritogenic peptide to, and expansion of, autoreactive T cell clones (see also 1.1.6.1.2). Commensurate with this T cells can be co-localized to MHC II expressing APC in the RA and PsA synovium (15, 273, 274). Furthermore, a large number of SM localized T cells are  $CD45RO^+$ , indicative of immunological memory and clonal expansion. Third, several murine models of arthritis, including CIA and adjuvant arthritis, are  $CD4^+$  T cell dependent (275, 276). Fourth, administration of T cell directed therapies, such as cyclosporine and leflunomide, have conferred considerable clinical benefit in RA and PsA (277-279). RA in particular, but also PsA, is considered to be a Th1 mediated disease due to a predominance of Th1 cytokines, although Th2 cytokines are also detectable (280-282) (see also 1.1.8.1). The mechanisms by which this occurs remain elusive, although the influence of direct cellular contact is believed to be a central mechanism of arthritic pathology.

In contrast to  $CD4^+$  T cells,  $CD8^+$  T cells, or cytotoxic T cells, mediate effector function by MHC I directed antigen specific killing of target cells and through cytokine production, in

**Figure 1.2 A proposed model of the collaboration of innate and adaptive immunity in the induction and amplification of chronic inflammatory arthritis.**

In this model stimulation of TLR ligands expressed by DC, M $\phi$  and fibroblasts can activate innate immune up-regulation of pro-inflammatory cytokines, chemokines and adhesion molecules facilitating leukocyte recruitment to the inflamed tissue. DC presentation of antigenic peptide/self (auto) antigen in the lymph node induces clonal expansion of autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells and B cell differentiation and autoantibody production. In the presence of pro-inflammatory cytokines and chemokines, fibroblast-like synoviocytes upregulate RANK-L expression activating osteoclast-mediated bone resorption and cartilage invasion. The subsequent release of necrotic cellular and matrix debris, as well as heat-shock proteins, can stimulate TLR ligands on APC of the innate immune system, thus eliciting amplification and perpetuation of inflammation in genetically susceptible individuals.

## Innate Immunity



particular IFN- $\gamma$  and TNF- $\alpha$ . Increasingly, a role for autoreactive CD8<sup>+</sup> T cells in disease progression in RA and PsA has been indicated. A population of highly expanded CD8<sup>+</sup> T cell clones with identical or highly homologous inferred CDR3 amino acid sequences, indicative of clonal expansion driven by the same antigenic peptide, has been identified in PsA SM (131). Furthermore these clones persisted during methotrexate treatment. Similarly, in RA clonally dominant CD8<sup>+</sup>CD57<sup>+</sup> T cells carrying T cell  $\beta$  receptors with identical amino acid sequences, indicative of clonal expansion driven by a common antigen, have been identified in unrelated patients (283-285). Whether these CD8<sup>+</sup> T cell clones are clonally expanded to autoantigen or not, has not yet been clarified. Of interest, studies have reported greater CD8<sup>+</sup> T cell enrichment to PsA SF than in RA SF and may reflect a more important role for CD8<sup>+</sup> T cells in the former as compared to the latter, commensurate with strong PsA MHC class I genetic and HIV associations (166, 286-288).

#### ***1.1.7.2.2 B cells***

B cells mediate adaptive immunity principally through the secretion of antigen-specific antibodies that promote pathogen elimination, but are also capable of antigen-presentation. Much evidence points to a key role for B cells in the perpetuation of arthritic pathology including the formation of germinal centres in the synovium (134), the clinical benefits of anti-CD20 (Rituximab) targeted therapy (289, 290) and the presence of autoantibodies. Of particular importance, it is increasingly recognised that B cells may be critical to the initiation of RA as autoantibodies directed against self-antigens including type II collagen, rheumatoid factor and cyclic citrullinated peptides (CCP) have been identified in the serum of RA patients many year before clinical manifestations of disease, consistent with an early breach in B cell tolerance (291-293). A similar finding is yet to be demonstrated in PsA.

#### ***1.1.7.2.3 Autoantigens in Inflammatory Arthritis***

It has been proposed that DC presentation of arthritogenic antigen to T cells, may drive memory T cell responses, promoting B cell activation and immunoglobulin class switching, hence playing a central role in the pathogenesis of inflammatory arthritis (273, 294-296). Commensurate with this, antibodies to denatured collagen were first detected in RA synovial effusions in 1978 (297). Numerous putative autoantigens have since been identified and confirmed, predominantly in RA, by the reactivity of antibodies in patient sera and through *in vitro* T cell proliferation assays (15). Joint-associated autoantigens

include human cartilage (HC) gp-39 (298, 299), collagen type II (CII) (300) and cartilage proteoglycan aggrecan (301). Elevated humoral immunity to proteoglycan decorin has also been observed in PsA (302). Autoantigens not associated with the joint include endoplasmic reticulum molecular chaperone immunoglobulin binding protein (BiP) (303-305), heterogeneous nuclear ribonucleoprotein A2 (RA33) (306, 307), glucose-6-phosphate isomerase (GPI) (308), citrullinated  $\alpha$ -enolase (309), citrullinated eukaryotic translational initiation factor 4G1 (eIF4G1) (310) and anti-cyclic citrullinated peptide (anti-CCP) (311). Anti-CCP antibodies may prove a useful serologic indicator for early RA diagnosis and prognosis as they are expressed in the inflamed joint, their presence precedes disease manifestation, and they are closely linked to PADI and HLA-DR susceptibility epitope polymorphisms (175, 312). However, although anti-CCP antibodies are considered highly specific markers for RA, they have also been identified in 7.9-12.5% of PsA patients with predominantly severe symmetrical arthritis (313, 314). Similarly, anti-GPI antibodies have been identified in PsA at comparable frequencies (12-25%) to those observed in RA (315). A number of studies support an autoantigen driven response in PsA (131, 316), however the associations identified to date are not strong. TCR  $\beta$ -chain nucleotide sequencing of PsA synovial tissue identified a small population of CD8 T cell clones with highly homologous CDR3 amino acid sequences, suggesting a common triggering autoantigen (131). Clearly PsA autoantigenic associations, or otherwise, require more resolution. Of interest, a number of strong candidate autoantigens have emerged in psoriasis and include keratin 13 (K13), heterogeneous nuclear ribonucleoprotein-A1 (hnRNP-A1) and rab coupling protein isoform 3 (FLJ00294) (RAB11FIP1) (317-319). It has been suggested that the lower plasma cell frequency in the PsA synovium as compared to the RA synovium may account, in part, for the less consistent autoantigen associations (127).

The role that candidate autoantigens play in the clinical manifestations of disease is, as yet, unclear. It has been suggested that in the proinflammatory synovial environment the DC phenotype may become skewed, resisting the immunosuppressive effects of IL-10 (295, 320) resulting in reduced chemotactic motility and an abnormally prolonged life span. Thus the persistence of DC-lymphocyte aggregates in synovium may contribute to the perpetuation of the inflammatory response (321). Of note, production of autoantibodies against the self-antigen glucose-6-phosphate isomerase (GPI) is believed to initiate

synovial inflammation in the K/BxN murine model of arthritis since sera transfer is sufficient to induce arthritis in naïve recipients (322-324). This model also supports the proposition that joint associated autoantigens may not be a pre-requisite for arthritis induction. However, the pathological manifestations of arthritis in the K/BxN model are also dependent upon an inflammatory cytokine cascade, particularly IL-1 and TNF- $\alpha$ , as demonstrated using cytokine knockout mice (325).

### **1.1.8 Mechanisms of inflammation**

The myriad inflammatory cells infiltrating the synovial compartment release soluble factors including cytokines, chemokines and matrix-metalloproteinases that are directly culpable for disease pathology by promoting recruitment and activation of further inflammatory cells.

#### **1.1.8.1 Cytokines**

The underlying aetiology of RA and PsA is not clear, however the critical importance of the cytokine cascade in the established phase of disease has been extensively studied. Cytokines are small (5 to 50 kDa), nonstructural proteins or glycoproteins that serve as chemical messengers between cells (326) and thus are of critical importance to virtually every aspect of innate and adaptive immunity including cell growth, differentiation, recruitment and activation, antigen presentation, tissue repair and remodeling and adhesion molecule expression. Cytokines are upregulated in inflammatory diseases such as RA and PsA, where they regulate the acute and chronic inflammatory response. Although cytokines can work in an autocrine fashion (act on the same cell), the proximity of cells in the synovium, particularly in the intimal lining layer enhances cytokine mediated inflammation by enabling paracrine (neighbouring cell activation) and juxtacrine (cell-to-cell contact) activity.

In both RA and PsA numerous pro-inflammatory cytokines including IL-1 $\alpha/\beta$ , IL-6, IL-12, IL-15, IL-17, IL-18, IL-32, GM-CSF and TNF- $\alpha$  are elevated in serum, synovial fluid and synovium (282, 327-332) (Table 1.2). Th1 cytokines, in particular those associated with highly activated macrophage frequency, are more prevalent in RA than in other forms of



**Table 1.2** Major cytokines with a role in inflammatory arthritis\*

Cytokine	Principal Source	Primary Biological Activity
<b><i>IL-1 Superfamily</i></b>		
IL-1 $\alpha$ /IL-1 $\beta$	Monocytes, M $\phi$ , DC B cells	$\uparrow$ COX-2, iNOS, PGE2 and nitric oxide synthesis, adhesion endothelial adhesion-molecule expression and collagenase by fibroblasts and chondrocytes.
IL-1Ra	Monocytes	Antagonize effects of IL-1 $\alpha$ and IL-1 $\beta$
IL-18	Monocytes, DC, PMN, endothelial cells	Pleiotropic: + IL-12 $\uparrow$ IFN- $\gamma$ production (Th1), + IL-4 (Th2)
<b><i>IL-6 Superfamily</i></b>		
IL-6	Fibroblasts, T cells, monocytes, M $\phi$ , B cells	Thrombopoiesis, cytotoxicity, inflammation, acute-phase reaction, haematopoiesis, bone metabolism. $\uparrow$ B cell proliferation and IgG <sub>1</sub> secretion.
<b><i>IL-10 superfamily</i></b>		
IL10	Monocytes, M $\phi$ , CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells, DC, epithelial cells.	$\downarrow$ IL-12, IFN- $\gamma$ , IL-2, TNF- $\beta$ , TNF- $\alpha$ in Th1, chemotactic for CD8 T cells; DC activation $\downarrow$ cytokine release; $\downarrow$ fibroblast MMP and collagen release; enhance B cell isotype switching.
<b><i>TNF Superfamily</i></b>		
TNF- $\alpha$	Monocytes, M $\phi$ , neutrophils, DC, activated T cells, NK cells, endothelial cells, fibroblasts	$\uparrow$ ICAM-1 and VCAM-1 and E-selectin, IL-1, IL-6, IL-8 and GM-CSF; neutrophils activation; mediates of toxic shock and sepsis.
RANK-L	Stromal cells, osteoblasts, T cells	Stimulates osteoclast mediated bone resorption; modulates T cell-DC interaction
LT $\alpha$	T cells, monocytes, fibroblasts, endothelial cells	Peripheral lymphoid development; similar bioactivities to TNF- $\alpha$ .
<b><i>Type II Interferon</i></b>		
IFN- $\gamma$	T cells, NK cells	$\downarrow$ bone resorption; $\downarrow$ T cell growth; opposes Th2; $\uparrow$ MHC I and II expression; M $\phi$ activation; $\uparrow$ DC and APC function; fibroblast collagen synthesis; $\uparrow$ endothelial adhesion molecule;
<b><i>Type I Interferon</i></b>		
IFN- $\alpha/\beta$	pDC, B cells	Activates naive CD4 <sup>+</sup> T cells, M $\phi$ ; enhances CD4 <sup>+</sup> production of IFN- $\gamma$ and IL-10; $\uparrow$ MHC; antiviral
<b><i>IL-17 family</i></b>		
IL-17	Th17, activated memory B cells	Bone and cartilage destructive factor; enhances leukocyte and fibroblast cytokine production.

APC, antigen-presenting cell; COX-2, cyclo-oxygenase type 2; DC, dendritic cell; GM-CSF, granulocyte macrophage colony stimulating factor; ICAM, intracellular adhesion molecule; Ig, immunoglobulin; IL, interleukin; IFN, interferon; iNOS, inducible nitric oxide synthase; MHC, major histocompatibility complex; M $\phi$ , macrophage; MMP, matrix metalloproteinase; NK, natural killer; PGE2, prostaglandin-E2; VCAM vascular adhesion molecule;  $\uparrow$  increase/enhance;  $\downarrow$  decrease/suppress.

\*Adapted from (327-330, 332, 333)

**Table 1.2** Major cytokines with a role in inflammatory arthritis (continued)\*

Cytokine	Principal Source	Primary Biological Activity
<i>4<math>\alpha</math>-helix family</i>		
IL-2	Activated Th1 cells, NK cells	↑ Proliferation and activation of T cells, B cells and NK cells, ↑ IFN- $\gamma$ and TNF- $\alpha$ .
IL-4	Th2 cells, NK cells, mast cells	↑ B cell proliferation, differentiation and MHC II expression, ↑ IgG <sub>1</sub> and IgE but ↓ IgM, IgG <sub>2a</sub> , IgG <sub>2b</sub> , IgG <sub>3</sub> , ↑ IL-1Ra expression, ↓ IL-1, IL-6 TNF- $\alpha$ and IFN- $\gamma$ .
IL-5	Th2 cells, NK cells, mast cells	↑ proliferation and activation of eosinophils, ↑ differentiation of cytotoxic T cells from thymocytes; B cell differentiation; IgA production.
IL-15	T cells, monocytes, M $\phi$ , DC	↑ T cell and NK cell recruitment, activation, proliferation, ↓ T cell death; ↑ neutrophils; + IL-12 ↑ IFN- $\gamma$ production; ↑ TNF- $\alpha$ production.
<i>IL-12 family</i>		
IL-12	Monocytes, M $\phi$ , DC, T cells, B cells	↑ NK cell and cytotoxic T cell activity; +IL-15/IL-18/IL-27 ↑ IFN- $\gamma$ production, ↑ TNF- $\alpha$ production.
IL-23	Monocytes, DC, M $\phi$	T cell proliferation and IFN- $\gamma$ production
IL-27	Monocytes, DC, M $\phi$ , endothelial cells	+ IL-12 ↑ IFN- $\gamma$ production; anti-tumour activity; clonal naïve CD4 <sup>+</sup> T cell expansion.
<i>Miscellaneous</i>		
IL-32	T cells, NK cells, FLS,	↑ TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and CXCL-1.
GM-CSF	T cells, M $\phi$ , DC, fibroblasts, endothelial cells	Granulocyte and monocyte maturation; haemopoietic effects; leukocyte PG release; DC maturation;
HMGB1	M $\phi$ , necrotic cells, widespread expression	Necrosis-induced inflammation; smooth muscle chemotaxis; bacteriocidal; disrupts epithelial barrier function; DNA-binding transcription factor; M $\phi$ activation and delayed proinflammatory cytokine.
TGF- $\beta$	Fibroblasts, monocytes, T cells, platelets	Initial activation then suppression of inflammation; ↓ T cell and NK cell proliferation and effector function; early phase leukocyte recruitment; wound repair and matrix maintenance; ↓ M $\phi$ iNOS.

APC, antigen-presenting cell; COX-2, cyclo-oxygenase type 2; DC, dendritic cell; GM-CSF, granulocyte macrophage colony stimulating factor; Ig, immunoglobulin; IL, interleukin; IFN, interferon; iNOS, inducible nitric oxide synthase; MHC, major histocompatibility complex; M $\phi$ , macrophage; MMP, matrix metalloproteinase; NK, natural killer; PGE<sub>2</sub>, prostaglandin-E<sub>2</sub>; RANK-L, receptor activator of NF- $\kappa$ B ligand; TGF, transforming growth factor; ↑ increase/enhance; ↓ decrease/suppress.

\*Adapted from (327-330, 332, 333)

arthritic disease (331, 334, 335), however elevated levels are also observed in PsA (280, 282). In addition, intracellular factors considered key regulators of cytokine production such as nuclear factor (NF)- $\kappa$ B, extra-cellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 are activated and expressed in RA synovial tissue (336, 337). The critical role of cytokines in the pathogenesis of RA and PsA is exemplified by the (varied) success of specific cytokine targeted therapies including anti-TNF- $\alpha$  (338), anti-IL-6 (339, 340) and anti-IL-15 (341, 342).

Of note, anti-inflammatory cytokines such as IL-4 and IL-10, as well as cytokine-inducible suppressor of cytokines (SOCS)-1 and -3 and IL-1 receptor antagonist (IL-1Ra) and soluble TNF-receptor (sTNFR) are also detectable in the inflamed synovial compartment (343). However, the levels of these are thought to be too low to be biologically effective, thus disequilibrium exists in the balance of pro- and anti-inflammatory signals. Alternatively, and given that cytokines can exert biological effects at picomolar concentrations, it may point to an inherent defect in the cells of the immune system to respond to anti-inflammatory signals by, for example, receptor deficiency as is the case for IL-10R $\alpha$  expression by RA derived fibroblasts (344).

#### **1.1.8.2 Chemokines**

The ingress of leukocytes to the inflamed synovium is mediated by chemokines. Chemokines, or chemotactic cytokines, are small molecular weight (8 to 12 kD) chemoattractant proteins that primarily promote angiogenesis, leukocyte recruitment and activation (345). Over 50 distinct chemokines have been identified and can be classified into four sub-families: CXC, which act primarily upon neutrophils but also promote angiogenesis (1), CC, which act primarily upon monocytes and T cells (2), C, which comprises XCL1 (lymphotactin) and XCL2 (single cysteine motif-1 $\beta$  (SCM-1 $\beta$ )) (3) and CX<sub>3</sub>C, which consists of the membrane bound fractalkine (CX3CL1) (4). A number of pro-inflammatory chemokines and chemokine receptors have been detected at elevated levels in both RA and PsA serum, synovial fluid and synovial tissue (Table 1.3), although RA levels generally exceed those observed in PsA, and both are higher than those observed in OA (346-361).

**Table 1.3** Major chemokines with a role in inflammatory arthritis\*

Systematic Name	Receptor (s)	Primary Source	Physiological Features
<b>CC chemokine family</b>			
CCL2 (MCP-1/MCAF)	CCR2	Fibroblasts	Inflammation
CCL3 (MIP-1 $\alpha$ /LD78 $\alpha$ )	CCR1, CCR5	Mononuclear cells, fibroblasts	Inflammation
CCL4 (MIP-1 $\beta$ )	CCR5	Monocytes, B cells, DC	Inflammation
CCL5 (RANTES)	CCR1, CCR5	CCR3, Fibroblasts, T cells, M $\phi$	Inflammation
CCL19 (MIP-3b/ELC)	CCR7	M $\phi$ , neutrophils	homeostatic
CCL20 (MIP-3 $\alpha$ /LARC)	CCR6	M $\phi$ and fibroblasts	Inflammation; homeostatic
CCL21 (6Ckine.SLC)	CCR7	Endothelial cells	homeostatic
<b>C Chemokine family</b>			
XCL1 (Lymphotactin)	XCR1	activated CD8 <sup>+</sup> T cells, NK cells, $\gamma\delta$ T cells, mast cells	Inflammation; Homeostatic (?)
XCL2 (SCM1- $\alpha$ )	XCR1	Mononuclear cells	Inflammation; homeostatic (?)
<b>CXC chemokine family</b>			
CXCL1 (MGSA- $\alpha$ )	CXCR2, CXCR1	Fibroblasts	Inflammation
CXCL5 (ENA-78)	CXCR2	Fibroblasts	Inflammation
CXCL8 (IL-8)	CXCR1, CXCR2	Widespread	Inflammation
CXCL10 (IP-10)	CXCR3	Monocytes, DC, epithelial cells, endothelial cells	Inflammation
<b>CX<sub>3</sub>C chemokine family</b>			
CXC3CL1 (Fractalkine)	CXC3CR1	Monocytes, M $\phi$ , fibroblasts, endothelial cells, DC	Inflammation

CCL, CC chemokine ligand; CCR, CC chemokine receptor; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; DC, dendritic cell; ELC, Epstein-Barr virus-induced receptor ligand; LARC, liver and activation-related chemokine; M $\phi$ , macrophages; MCAF, Monocyte Chemotactic and Activating Factor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; NK, natural killer; RANTES, regulated on activation normal T cell expressed and secreted; XCL, C ligand; 6Ckine.SLC, 6Ckine-secondary lymphoid tissue chemokine.

\* Adapted from (346-362)

### 1.1.9 Current therapies in PsA and RA

Current biologic response modifying therapies represent significant advances, combining improved efficacy with reduced toxicity, as compared to traditional drugs such as sulphasalazine, cyclosporine, methotrexate, NSAIDs and corticosteroids (363). In particular the effects of blocking TNF- $\alpha$  using adalimumab (a human anti-TNF- $\alpha$  monoclonal antibody), infliximab (a chimeric anti-TNF- $\alpha$  monoclonal antibody) or etanercept (soluble TNF- $\alpha$  receptor-IgG<sub>1</sub> fusion protein) have been dramatic in reducing disease activity, retarding or halting radiographic erosions and inhibiting subsequent joint damage hence improving functional disability, particularly when used in combination with methotrexate (364-368). In addition, adalimumab has been shown to reduce rheumatoid factor and anti-CCP antibody titres (369). Numerous adverse medical side effects including exacerbation of multiple sclerosis (370), inducement of SLE-like disease (371), rapid onset of non-melanoma skin cancer (372) and increased susceptibility to infections from, for example, *Listeria monocytogenes* (373, 374), *Salmonella typhimurium* and *Candida albicans* (375), have been reported. Such severe adverse side effects are however rare, and incidences may not exceed rates expected in the general population (376). Nevertheless, many patients do not respond to therapy and in those that do 'true' remission, whereby patients are able to discontinue medications with no clinical or radiological evidence of damage, is rare. This is most likely because current treatment strategies target the cytokines and enzymes directly culpable for disease pathology, therefore focusing on disease management rather than cure.

Several lines of research have implicated the participation of T cells in the pathogenesis of arthritis. These include the recruitment of autoreactive T cells to the inflamed synovium (377, 378), the strong genetic association of selected HLA-DR (MHC class II) haplotypes with disease susceptibility (160, 379, 380) and the T cell dependency of several animal models of arthritis, including collagen induced arthritis (CIA) and adjuvant arthritis (15). The mechanisms triggering the aberrant T cell activity have, however, been only partially characterised and include cytokine bystander activities. The critical challenge in therapeutics now is to uncover the underlying immunological processes responsible for the generation and perpetuation of inflammatory arthritis such that peripheral tolerance can be re-established and therapeutic remission can be maintained. The recent advent of anti-CD20 (Rituximab) and CTLA4-Ig (Abatacept), which inhibits the costimulation between

B7 on antigen presenting cells (APC) and CD28 on naive T cells, as a treatment for rheumatoid arthritis (RA) (381) suggest that such effects may be achievable.

Of particular interest are the co-stimulatory interactions bridging the innate and adaptive immune response, notably those initiated by dendritic cell (DC) interactions with T cell subsets, as the evolution of the latter is profoundly influenced by these cognate interactions. Because DC are the only APC recognised to date as capable of provoking autoimmune disease (382), they represent an important line of investigation in determining the initial triggers that generate and potentially maintain chronic inflammatory arthritis.

## **1.2 Dendritic Cells**

Arising from CD34<sup>+</sup> haematopoietic progenitors, DC were first described in the 1970s by Ralph M. Steinman and Zanvil A. Cohn, who characterised a novel mononuclear leukocyte with distinctive dendritic cytoplasmic extensions (383-386). Termed dendritic cells, they have since been identified in blood, all lymphoid tissues and the majority of non-lymphoid tissues (384, 387-394). DC are a highly motile complex network of professional antigen-presenting cells (APC) critical to the initiation and regulation of adaptive immunity against pathogens and tumours, as well as the maintenance of both central and peripheral tolerance (395). Morphologically, DC have a characteristic pattern of cell processes, including spiny dendrites, bulbous pseudopods and lamellipodia or veils (396). There is however, considerable phenotypic, morphologic and functional variation between the DC subsets related to lineage, state of maturation and tissue localisation.

### **1.2.1 Dendritic Cell Lineage**

Three distinct stages of DC development have been identified. The first stage, generated from CD34<sup>+</sup> bone marrow progenitors, are the circulating precursor peripheral blood (PB) DC, of which six distinct subsets of lineage negative (CD3, CD14, CD20 and CD56) and HLA-DR<sup>+</sup> DC have been delineated in humans. Four of these subsets, CD1b/c<sup>+</sup>, CD16<sup>+</sup>, CD141 (BDCA-3)<sup>+</sup> and CD34<sup>+</sup>, are myeloid derived, while the remaining two, CD123<sup>+</sup>/CD303 (BDCA-2)<sup>+</sup>, have a lymphoid phenotype, and are described as plasmacytoid DC (pDC) (397, 398) (Table 1.4). Recently, pDC have been delineated into two distinct subsets based on the differential expression of CD2. The more common, CD2<sup>-</sup>

**Table 1.4** Phenotype of human DC subsets\*

	Myeloid DC	Plasmacytoid DC
<i>Phenotype</i>		
HLA	+	+
CD1a/b/c/d	+/-	-
CD2	-	-/+
CD4	+	++
CD11b	+	-
CD11c	++	-
CD13	+	-
CD14	-	-
CD16	+/-	-
CD33	+	-
CD45RA	-	+
CD45RO	+	-
CD123	+	+++
CD141 (BDCA-3)	+/-	-
CD303 (BDCA-2)	-	+
CD304 (BDCA-4)	-	+
Ig $\lambda$ -like 14.1	-	+
Pre-T $\alpha$	-	+
Spi-B	-	+

\* Adapted from (395, 397, 399, 400)

pDC comprise 75% of all circulating pDC. It is thought that the less frequent, CD2<sup>+</sup> pDC, are able to kill target cells (K562) and express lysozyme. Both subsets however are capable of potent type I interferon (IFN) release and induce proliferation of memory T cells (400).

It is important to note that in addition to the six 'classical' circulating DC progenitors, a number of other cell types have been identified that are capable of differentiating into DC-like cells. Prominent amongst these are the CD14<sup>+</sup> monocytes, which when matured with GM-CSF and IL-4 or TNF- $\alpha$  are able to differentiate into mDC, down-regulating CD14 and up-regulating CD11c (395). Monocyte-derived (mo) DC also attain the majority of described mDC functions and as such, due to their greater frequency in blood and ease of culture in bulk, are commonly utilised as substitutes for "true" mDC. Cautionary to this use is a report by Osada et al., who observed that peripheral blood DC enhanced NK cell activity in assays of anti-tumour cytotoxicity, but monocyte-derived DC did not (401). Moreover, monocytes do not express endosomal toll-like receptor (TLR) 3 whereas mDC do, allowing recognition of double-stranded (ds) RNA virus (402). Monocyte differentiated DC therefore represent a pragmatic, but flawed, substitute for analysing DC function. Other cells may also acquire or possess APC activity.

It has been demonstrated that isopentenyl pyrophosphate (IPP) or *Escherichia coli* stimulation induces  $\gamma/\delta$  T cells to acquire not only antigen presenting phenotype, with up-regulation of MHC-II, CD40, CD80 and CD86, but more significantly function, with the ability to process and present soluble protein antigen to CD4<sup>+</sup>  $\alpha\beta$  T cells (403). Moreover, the level of antigen presentation and co-stimulation receptor expression was at directly comparable levels to LPS stimulated monocyte-derived DC.

In addition to  $\gamma/\delta$  T cells it has recently been reported that in a pro-inflammatory environment NK cells can become antigen-presenting cells (404), with up-regulation of MHC-II and T cell costimulatory molecule expression, and acquisition of the functional ability to present target cell-derived antigens following cytotoxic killing. It is probable that the recently described interferon-producing killer dendritic cells (IKDC), which are B220<sup>+</sup>/Ly6c<sup>-</sup>/NK1.1<sup>+</sup>/GR-1<sup>+</sup>/PDCA-1<sup>+</sup>/CD122<sup>+</sup>/MHC-II<sup>+</sup>, are the murine equivalent of these cells, although this is yet to be formally established. Possessing the molecular profile



of both NK cells and DCs, they produce type I interferons and IL-12 or IFN- $\gamma$  (stimuli dependent). IKDC have the same cytolytic capacity of regular NK cells but rapidly lose this ability along with the loss of NKG2D receptor, and concomitant gain in DC-like APC activity and up-regulation of MHC-II and co-stimulatory molecules, features which distinguish them from regular NK cells (405-407). Furthermore, IKDC have been proposed to constitute a third distinct DC lineage, as although (in the murine system) they display MHC-II at comparable levels to pDC, they do not display the pDC receptor mouse plasmacytoid dendritic cell antigen (mPDCA)-1.

The extent to which  $\gamma/\delta$  T cells and NK cells can differentiate into DC, similar to monocytes, or purely gain aspects of DC-like function, requires further resolution. Nevertheless, these studies may indicate an intriguing system of circulating pre-cursor DC comprising not only mDC and pDC, but also monocytes,  $\gamma/\delta$  T cells and NK cells (Figure 1.3) (400). Significantly, these are all cells of integral importance to maintaining innate immunity. I will now focus on the well-established, defined 'classical' DC subsets, mDC and pDC respectively.

#### **1.2.1.1 Myeloid Dendritic Cells**

Myeloid DC (mDC) are phenotypically characterised as CD1c<sup>+</sup>/CD11c<sup>+</sup>/CD45RO<sup>+</sup>/HLA-DR<sup>+</sup>/CD123 (IL-3R $\alpha$ )<sup>lo</sup> (408). Comprising 0.5-1.0% of all circulating mononuclear cells, mDC express the myeloid markers CD13 and CD33 reflecting a lineage relationship in common with both monocytes and macrophages (395). Capable of IL-12p70, IL-23, IL-15, IL-18, IL-1, IL-6, TNF- $\alpha$  and IL-10 production as well as antigen capture and presentation (409), mDC require GM-CSF for survival *in vitro* and can differentiate into CD1a<sup>-</sup> interstitial DC and CD1a<sup>+</sup> Langerhans cells in the presence of GM-CSF, IL-4 and TGF- $\beta$  (410). Triggering by dsRNA viruses also triggers the production of a small amount of type I interferons (399).

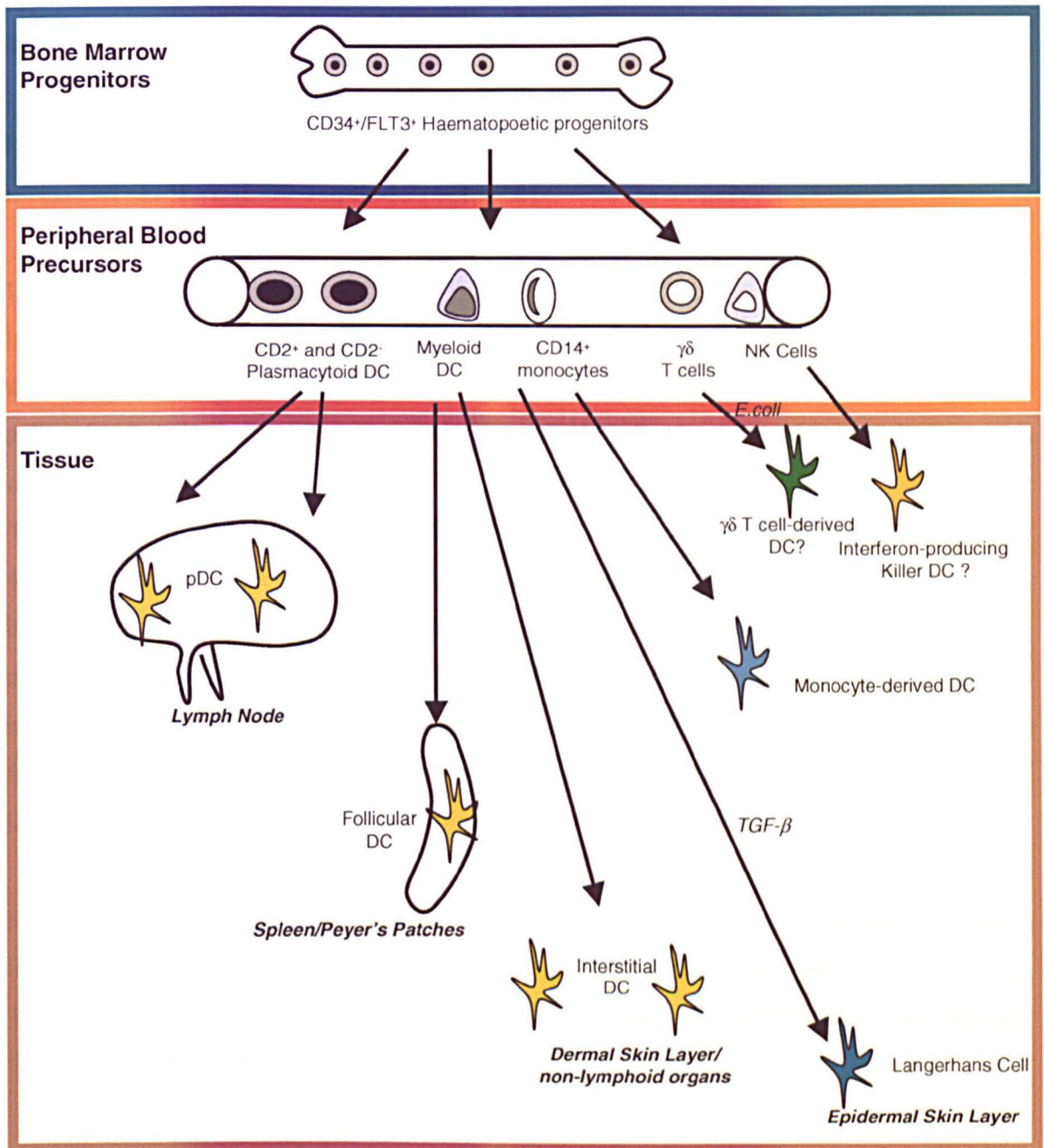
#### **1.2.1.2 Plasmacytoid Dendritic Cells**

Lymphoid lineage plasmacytoid DC (pDC) have been variously described as plasmacytoid T cells (1983), plasmacytoid monocytes (1988) and type I interferon (IFN) producing cells (1970s), based upon their plasma cell-like ultrastructural features, including a round nucleus and basophilic cytoplasm, expression of MHC II and propensity to secrete large

### Figure 1.3 Human dendritic cell subsets\*

Bone marrow  $CD34^+$  progenitors differentiate into blood  $CD2^{+/-}$  plasmacytoid (p) DC, myeloid (m) DC,  $CD14^+$  monocytes,  $\gamma\delta$  T cells and NK cells. In the presence of growth factors such as GM-CSF and IL-4 monocytes can differentiate into Langerhans cells, which reside in the epidermis, or monocytes-derived (Mo) DC. pDC preferentially migrate direct from the peripheral blood to the lymph nodes. mDC migrate from the blood to the tissues, where they act as the body's sentinels, from there migrating to the lymph nodes through the afferent lymphatics.  $\gamma\delta$  T cells and NK cells may acquire antigen-presenting functions following antigen insult such as from *E. coli*.

\*Adapted from (401)



amounts of the type I interferons (IFN), 70-1000 times that of other cells, upon viral stimulation (399, 411, 412). The low frequency of pDC in PB, less than 0.3% of all circulating mononuclear cells (29) (33, 34), has confounded their classification. It was not until the late 1990's that pDC were formally defined as a distinct and unique dendritic cell subset, identified as CD303 (BDCA-2)<sup>+</sup>/CD304 (BDCA-4)<sup>+</sup>/CD123 (IL-3Rα)<sup>high</sup>/CD11c<sup>-</sup>/HLA-DR<sup>+</sup>/CD45RA<sup>+</sup>, lacking both CD13 and CD33 but expressing the lymphoid specific transcripts preT-cell receptor α, immunoglobulin (Ig) λ-like 14.1 and Spi-B (412-415). Requiring IL-3 for growth and survival *in vitro*, pDC can secrete, in addition to the type I IFNs, TNF-α, IL-6, IL-10 and IL-15 (411, 412).

### 1.2.1.3 Murine DC subsets

In the mouse, up to six DC subtypes have been identified and can be distinguished by CD4 and CD8 expression (Table 1.5). Three distinct myeloid CD11c<sup>hi</sup> DC subtypes have been delineated in spleen, CD4<sup>-</sup>/CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>-</sup>/CD8<sup>-</sup>. Lymph nodes contain two extra subtypes thought to have migrated from the tissue to the lymph nodes, CD4<sup>-</sup>/CD8<sup>-</sup>/CD205<sup>lo</sup>/CD11b<sup>+</sup> interstitial DC and CD4<sup>-</sup>/CD8<sup>lo</sup>/CD205<sup>hi</sup>/langerin<sup>+</sup> langerhans cells (416-418). In recent years a CD4<sup>-/+</sup>/CD8<sup>-/+</sup> plasmacytoid DC subset has been identified and can be distinguished from myeloid DC by CD11c<sup>lo</sup>/MHC II<sup>lo</sup>/B220<sup>hi</sup>/120G8<sup>+</sup>/mPDCA-1<sup>+</sup> expression (419-422). Murine DC subsets are remarkably phenotypically similar to their human counterparts, with the exception of CD8, although no evidence suggests this is functionally significant. In addition murine, but not human, pDC express CD11c, albeit at low levels.

Murine DC subsets share the majority of their functional features with human DC subsets, in particular type I IFN secretion from pDC and IL-12 secretion predominantly by mDC. The remainder of this review will focus upon the human DC subsets only, in particular their function with regard to maturation status.

## 1.2.2 Immature Dendritic Cells

### 1.2.2.1 Tissue Localisation

Migration of precursor DC from PB into tissue induces differentiation to an immature state. Immature tissue mDC can be further classified according to anatomic location and

**Table 1.5** Phenotype of murine DC subsets\*

	CD4 <sup>-</sup> CD8 <sup>+</sup>	CD4 <sup>-</sup> CD8 <sup>-</sup>	CD4 <sup>+</sup> CD8 <sup>-</sup>	CD4 <sup>-</sup> CD8 <sup>hi</sup>	CD4 <sup>-</sup> CD8 <sup>lo</sup>	pDC
<i>Phenotype</i>						
CD8	+	-	-	hi	lo	+/-
CD4	-	-	+	-	-	+/-
CD11b	-	+	+	+	+	-
CD11c	hi	hi	hi	hi	hi	lo
B220	-	-	-	-	-	+
Ly6c	-	-	-	-	-	+
CD45RB	+	+	+	+	+	+++
DEC-205	+	+/-	-	hi	lo	-
120G8	-	-	-	-	-	+
mPDCA-1	-	-	-	-	-	+

\*Adapted from (399, 423, 424)

subsequent functional niche (Figure 1.2). Langerhans cells, identified by the expression of the lectin langerin (CD207) (425) and presence of Birbeck granules (426) reside in the epidermal layer of the skin, while interstitial DC expressing intracytoplasmic transglutaminase clotting factor XIIIa (427) and the lectin DC-SIGN (428) reside in the dermal layer (383). Interstitial DC are also located in the interstitial connective tissues of non-lymphoid organs (429), while follicular DC are found in the B cell areas of the spleen and Peyer's patches (430).

Originally Langerhans cells were thought to be *the* DC subset responsible for communicating antigen from the skin to naïve T cells in the cutaneous lymph nodes (LN). Recent advances however have revealed that interstitial DC residing in the dermis precede Langerhans cells to the cutaneous LN following exposure to antigen, migrating within hours as compared to 3 days for Langerhans cells (431). It has subsequently been suggested that Langerhans cells have evolved to present antigen *in situ* to effector or memory T cells, or alternatively, to respond to pathogens for which a delayed migratory response is beneficial (432).

While circulating PB mDC enter peripheral tissues, migrating to the lymph nodes via the afferent lymphatics (395, 433, 434), circulating PB pDC are recruited directly to lymphoid tissues through high endothelial venules (HEV) in a CD62L, CXCL9 and CD62E dependent manner (435, 436). To date pDC have been identified in the spleen and T cell areas of the secondary lymphoid tissues (395, 412). In the steady-state pDC are predominantly absent from sites of pathogenic entry to the body, however they have been identified infiltrating sites of inflammation including the dermal layer of the skin in such conditions as psoriasis (437), cutaneous lupus erythematosus lesions (438) and lesional Lichen planus (439). The contrasting migratory properties of the precursor DC subsets result from differential CC chemokine receptor (CCR) expression.

#### **1.2.2.2 Chemokine Receptor Expression**

The ability of immature DC to home to sites of inflammation and antigen is an integral component to their function as APC. This migratory capacity is enabled by high expression of the proinflammatory chemokine receptors CCR1, CCR2, CCR5, CCR6 (Langerhans cells only), and CXCR1 which respond to inflammatory chemokines such as macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), monocyte chemotactic protein-1 (MCP-1)

and regulated on activation normal T cell expressed and secreted (RANTES) (440, 441). Although pDC and mDC display many of the same CCRs, their distinct patterns of migration can be attributed to differing expressions of key receptors (Table 1.6).

Compared to precursor mDC, precursor pDC express higher levels of CCR5, CXCR3 and significantly, the pivotal chemokine receptor driving DC migration to the lymph node, CCR7 (442). It is consequently thought that in the steady state, pDC comprise the most abundant DC population in lymphoid organs. Moreover, pDC have been identified as the dominant cytokine producing DC subset within the human lymph node (443). The rapid infiltration of pDC to sites of inflammation is also directed by the expression of serpentine chemokine-like receptor 1 (CMKR1/ChemR23), the ligand for the chemotactic agent chemerin, which is activated during blood coagulation (444), and expressed selectively by HEV in secondary lymphoid organs and inflamed endothelium (445).

The migratory capacity of DC, coupled with their propensity for antigenic capture make immature DC vital sentinels of the periphery.

### 1.2.2.3 Antigen Capture

Immature mDC are able to efficiently capture and process antigens at ambient picomolar and nanomolar concentrations via macropinocytosis (446), receptor-mediated endocytosis via C-type lectin receptors (mannose receptor, DC-SIGN, DEC-205, langerin) or Fc $\gamma$  type I (CD64) and II (CD32) receptors, and phagocytosis of particles such as apoptotic and necrotic cell fragments via CD91,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$  integrins and the thrombospondin receptor CD36 (447, 448). Furthermore, immature DC can internalise peptide loaded heat shock proteins (Hsp) gp96 and Hsp70 (395).

While the ability of mDC to uptake antigen has been convincingly established, the antigen-capturing competence of pDC is in debate. *In vitro* studies comparing the capacity of mDC and pDC subsets to phagocytose apoptotic and necrotic K562 leukaemia cells revealed no evidence of phagocytosis by pDC (449). However other studies have suggested that the capacity for phagocytosis and pinocytosis is intact in pDC, albeit weak in comparison to mDC (450, 451). Furthermore, pDC express a unique C-type lectin receptor, CD303 (BDCA-2), which may act as an antigen-capturing molecule (413). In

**Table 1.6 CC Chemokine Receptor expression on mDC and pDC\***

	Myeloid	Plasmacytoid
Immature	CCR2 <sup>high</sup> CXCR4 <sup>high</sup> CCR3 <sup>lo</sup> CCR4 <sup>lo</sup> CXCR1 <sup>lo</sup> CXCR2 <sup>lo</sup> CCR5 <sup>lo</sup> CCR7 <sup>lo</sup> CXCR3 <sup>lo</sup> CCR6 (Langerhans cells only)	CCR2 <sup>high</sup> CXCR4 <sup>high</sup> CCR3 <sup>lo</sup> CCR4 <sup>lo</sup> CXCR1 <sup>lo</sup> CXCR2 <sup>lo</sup> CCR5 <sup>high</sup> CCR7 <sup>high</sup> CXCR3 <sup>high</sup> CMKR1 (ChemR23) <sup>high</sup> CCR1 <sup>lo</sup>
Mature	CCR2 <sup>lo</sup> CCR5 <sup>lo</sup> CXCR3 <sup>lo</sup> CXCR4 <sup>lo</sup> CCR7 <sup>high</sup>	CCR2 <sup>lo</sup> CCR5 <sup>lo</sup> CXCR3 <sup>lo</sup> CXCR4 <sup>lo</sup> CCR7 <sup>high</sup>

CCR, CC chemokine receptor; CXCL, CXCR, CXC chemokine receptor; CMKR1, serpentine chemokines-like receptor 1.

\* (395, 442-444)



addition to this, CpG oligodeoxynucleotide (ODN) and influenza virus matured pDC are capable of stimulating antigen-specific memory recall T cell expansion and, in the case of influenza virus, antigen-specific primary and secondary CD4<sup>+</sup> and CD8<sup>+</sup> T cell expansion and differentiation *in vitro* (422, 452-456). Thus pDC function may be antigen and context dependent such that comparable studies with mDC *in vitro* are rendered meaningless.

#### **1.2.2.4 Antigen Processing**

Immature mDC and pDC produce large amounts of MHC class II molecules, localised to MHC class II-rich late-endosomal compartments, that can be loaded with captured antigenic peptide ready for transport to the cell surface for presentation (457-462). MHC II gene expression is controlled by the non-DNA binding coactivator MHC class II transactivator (CIITA), which in turn is regulated by three independent promoters pI, pIII and pIV (463). The promoter pI regulates CIITA expression in all myeloid lineage APC, including mDC, whereas the promoter pIII strictly regulates APC of lymphoid origin including pDC (464). It is likely that these differences impact fundamentally on the antigen presentation capabilities of the two subsets, particularly as cellular maturation and up-regulation of surface MHC II triggers pI mediated shutdown of MHC II biosynthesis in mDC but not pDC maturation (464).

In the steady state, antigen engulfment is thought to induce a tolerogenic immature DC phenotype characterised by low CD86 and HLA-DR expression, but increased expression of the lymph node homing CCR7. It is in the lymph node that these immature DC can induce tolerance. In contrast, a pro-inflammatory microenvironment, characterised by cytokines such as TNF- $\alpha$  and IL-1 $\beta$  and toll-like receptor (TLR) triggering ligands such as LPS and bacterial DNA, induces *en masse* DC migration and differentiation (465).

Whether immature DC, encountering antigen in the absence of danger signals, or non-activated mature DC are responsible for the induction of tolerance – and hence represent a tolerogenic DC phenotype is a matter requiring clarification.

#### **1.2.2.5 Differential Pattern-Recognition Receptor Expression**

DC express germ-line-encoded receptors known as pattern-recognition receptors, which include toll-like receptors (TLR), C-type lectins (for example DC-SIGN), NOD (for

example NOD2) and Mannose receptors. Pattern-recognition receptors recognise different pathogen-associated molecular patterns (PAMPS) associated with (for example) bacterial, viral, protozoan or fungal infection, playing a crucial role in the early phase of antigen uptake and subsequent immune response. It has been suggested that pDC and mDC may have evolved to combat different types of pathogens based upon their differential expression of pathogen-recognition receptors (Table 1.7). Similar subset propensity for antigen may be based on physiochemical properties. Thus the preferential expression by mDC of TLR1, 2, 4 and 6 makes them particularly suited for stimulation by lipid-based PAMPs. Similarly, numerous reports indicating that pDC have an aptitude for the presentation of endogenous antigens and viral antigens may be explained by their selective expression of intracellular TLR7 and TLR9.

### **1.2.3 Mature Dendritic Cells**

TLR, NOD or lectin signalling modulates pleiotropic effects on immature DC evolved to best facilitate the transition from innate recognition to an adaptive immune response and as such constitutes one of the pivotal control points of immunity and potentially autoimmunity. Cytoskeletal re-modelling is accompanied by down-regulation of the inflammatory chemokine receptors, but upregulation of the lymphoid homing receptors CCR4, CXCR4 and CCR7 (466) (Fig. 1.4). The maturing DC then migrate to lymphoid tissue via the lymphatics in response to secondary lymphoid tissue chemokine (SLC), produced by lymphatic endothelial cells, and Epstein-Barr virus-induced ligand chemokine (ELC), produced by stromal cells and DCs in T cell aggregates (395, 408, 466). Macrophage-derived chemokine (MDC), thymus and activation-regulated chemokine (TARC), pulmonary and activation-regulated chemokine (PARC) and IFN- $\gamma$ -inducible protein (IP-10) produced by mature DC in the lymph nodes, further enhance migration and promote DC to interdigitate to T cell areas of the lymph node (396). During this differentiation process, DC down-regulate the ability to capture and process antigen, but up-regulate the molecules required for efficient antigen presentation including peptide-loaded MHC I and II molecules, the costimulatory molecules CD80, CD86 and CD58 and signalling molecules CD40 and TRANCE/RANK-L (395). Thus mature DC are efficiently equipped to present antigen to T cells, generating antigen-specific effector lymphocytes that migrate back to the site of inflammation, hence initiating the adaptive immune

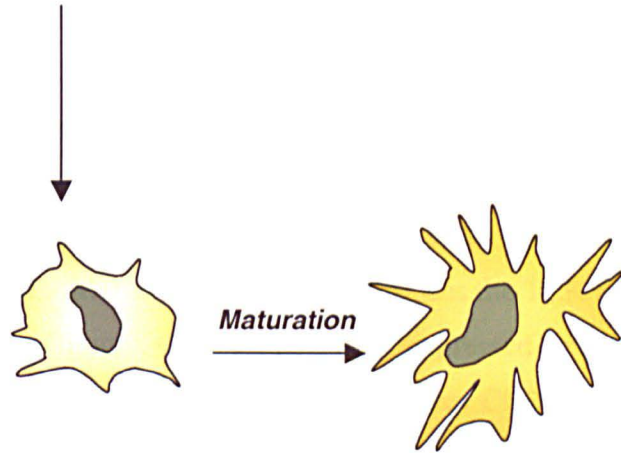
**Table 1.7** Toll-like Receptor expression by DC and their specific agonists.

	mDC	pDC	Ligand
TLR1	+	+	Forms heterodimers with TLR2 to detect pam3cys(467)
TLR2	+	-	Peptidoglycan (PGN)(468), mycoplasma lipoprotein (MALP)(469), HMGB1(470)
TLR3	+	-	DsRNA, polyriboinosinic:polyribocytidylic acid (poly(I:C))(471)
TLR4	+	-	Lipopolysaccharide (LPS)(472), Lipid A analogs(473), mycobacterial components(474), fibrinogen(475), surfactant(476) protein-A(477), heparan sulphate(478), soluble hyaluronan(479), $\beta$ -defensin 2(480), highmobility group box 1 protein (HMGB1)(470), Hsp60(481), Hsp70(482) Gp96(483)
TLR5	-	-	Bacterial flagellin(484)
TLR6	+	+	Lipid based PAMPs(485), required for TLR2 signalling recognition of PGN(486)
TLR7	+	+	Imidazaquinolines (imiquimod, R848) and guanosine analogs (Loxoribin)(487), ss RNA, GU-rich ssRNA (488, 489)
TLR8	+	-	ss RNA, GU-rich ssRNA, R848 (488, 489)
TLR9	-	+	Bacterial unmethylated deoxy-cytidylate-phosphate-deoxy (CpG) DNA (490)
TLR10	-	+	??

#### **Figure 1.4 Dendritic cell maturation**

Signalling immature dendritic cells (DC) through pattern-recognition receptors, CD40L or soluble factors such as TNF- $\alpha$  induces maturation. Maturation is accompanied by cytoskeletal re-modelling, down-regulation of antigen up-take but up-regulation of antigen presentation and MHC II expression. In addition mature DC up-regulate co-stimulation and maturation receptor expression and pro-inflammatory cytokine release as well up-regulating lymph node homing chemokines receptor expression. As such maturation best facilitates the translation from innate recognition to adaptive immunity.

TLR ligation/TNF- $\alpha$ /CD40L



Antigen Uptake	+++	+
Antigen Processing	+++	+
MHC II	+	+++
CD40/CD80/CD86	low	high
CD83/DC-LAMP	-	+
IL-12/TNF- $\alpha$	low	high
IFN- $\alpha$ (pDC)	high	low
CCR1/CCR5/CXCR1	high	low
CCR4/CXCR4	low	high
CCR7 (pDC)	high	high
CCR7 (mDC)	low	high
CXCR3 (pDC)	high	low
Inflammatory chemokines		Homeostatic chemokines

response. The direction in which the response is skewed is, however, dependent upon DC lineage, the maturation stimulus and the inflammatory mediators present at the site of infection (491).

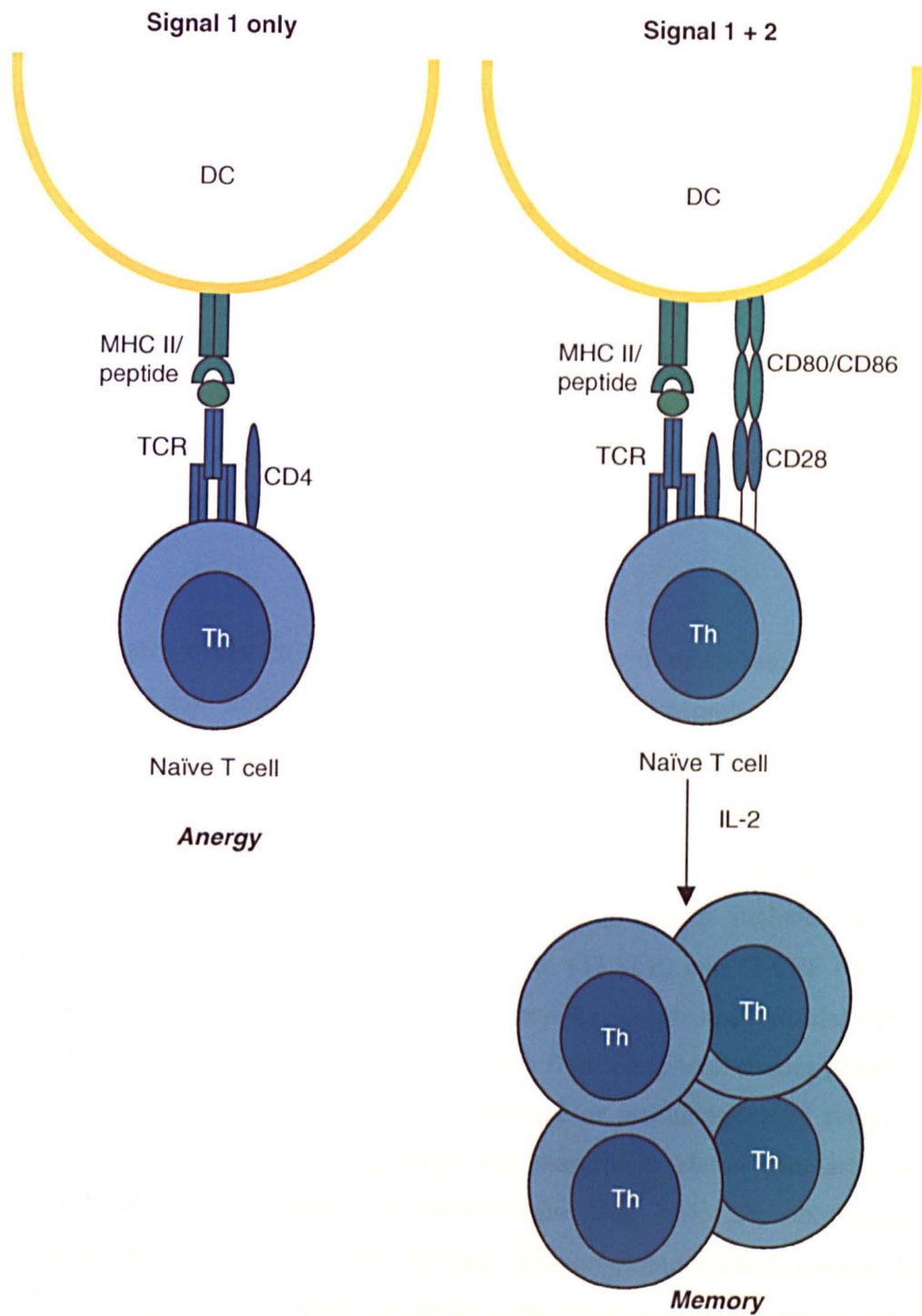
### **1.2.3.1 Interactions between DC and T cells**

The traditional paradigm for T cell activation by DC is based upon a bimodal model. Interaction of antigen-specific TCR with the MHC II peptide complex on DC induces T cell anergy (Signal 1). In order to generate T cell activation, IL-2 production and immunity, additional sustained co-stimulation by CD28/CD80/CD86 is required (Signal 2) (492) (Fig. 1.5). Optimal T cell activation is achieved by formation of an immunological synapse composed of a ring of LFA-1/ICAM-1 interactions that stabilises the TCR/MHC II and CD28/CD80/CD86 complex (493). Of note, CTLA-4 (CD152), expressed by activated T cells, has 10-20 times greater affinity to CD80/86 binding than does CD28. As such CTLA-4/CD80/CD86 is one critical mechanism by which the T cell response is modulated, exemplified by lethal lymphoproliferative disease in CTLA-4 knockout mice (494, 495). Additional modulation is likely provided by DC expression of the B7 family members ICOS-L and the inhibitory receptor programmed cell death receptor-1 (PDCD-1) (496, 497).

The differentiation and expansion of activated CD4<sup>+</sup> T cells into Th1 or Th2 cells is dependent upon additional DC signalling and soluble factors in the micro-environment. Th1 cells release pro-inflammatory cytokines including IFN- $\gamma$ , enhance macrophage cytokine release (IL-6, TNF- $\alpha$ ) and are important for cellular immunity. In contrast Th2 cells release anti-inflammatory cytokines, IL-4 and IL-5, and promote humoral immunity. Initial investigations suggested that mDCs and pDCs possessed an intrinsic ability to polarise naive T cells towards a Th1 or Th2 phenotype respectively. This observation was largely based upon the mDC secretion of IL-12, but pDC secretion of IFN- $\alpha$ , following CD40L stimulation (412). However, it is now recognised that while pDC prime T cells to secrete IL-4 (type 2) following incubation with IL-3 and CD40-L, they can also induce T cells to produce IFN- $\gamma$  (type I) and IL-10 (regulatory T cells) when given a viral stimulus (498). Moreover, pDC can (in common with mDC), produce high levels of IL-12p70 in response to CD40 ligation when accompanied by bacterial CpG motif stimulation (402, 499).

### **Figure 1.5 The two signal model of T cell activation by dendritic cells**

According to the traditional paradigm of T cell activation by dendritic cells (DC), antigen-specific T cell receptor (TCR) interacts with the MHC II peptide complex (Signal 1), which alone induces T cell anergy. T cell activation is generated by additional sustained signalling by CD28/ CD80/CD86 (Signal 2).





Thus the capacity of DC to initiate a given immune response does not appear to be an intrinsic quality, but rather a consequence of local environmental pressure.

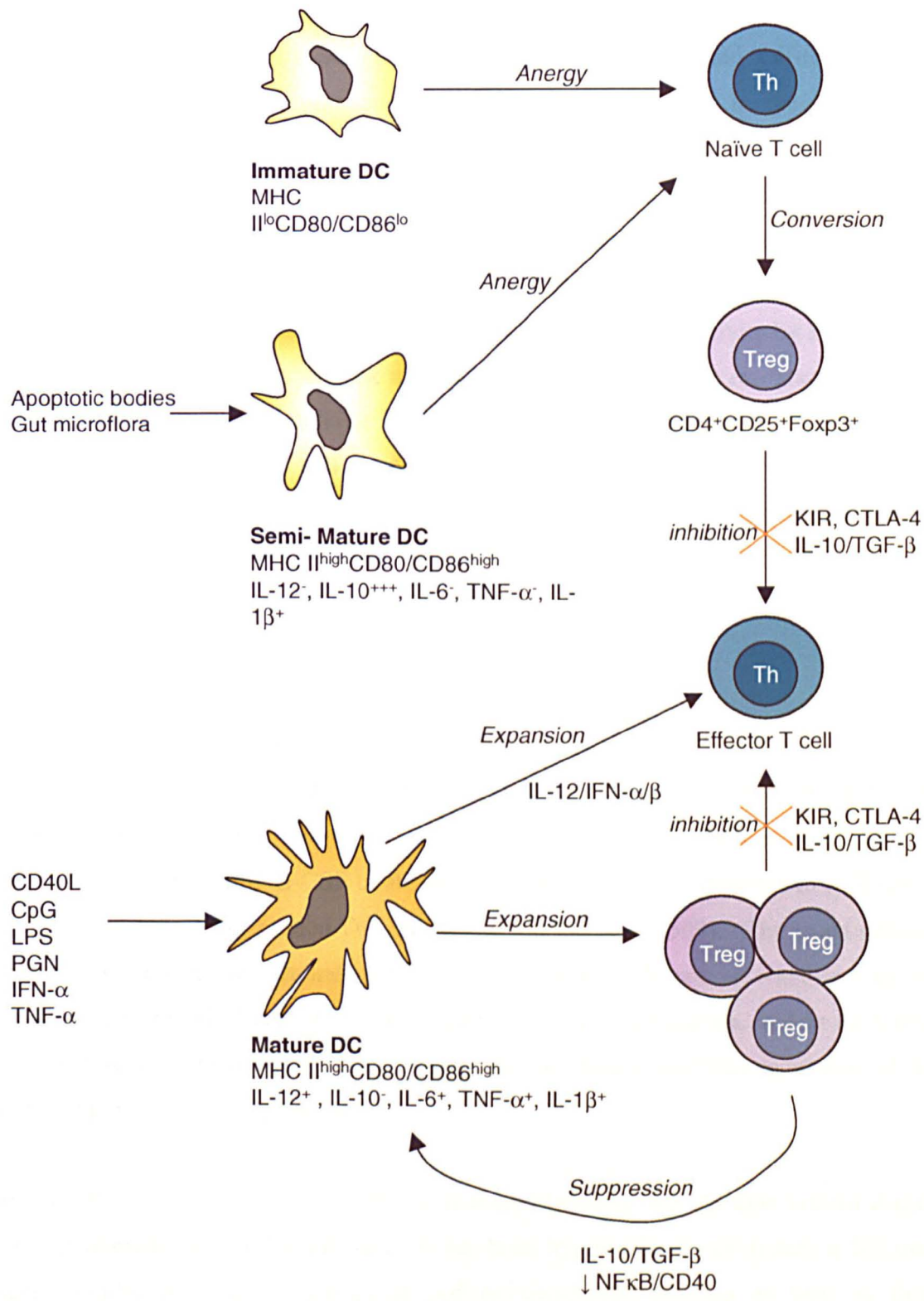
#### **1.2.4 Tolerogenic DC**

The development of central tolerance in the thymus, whereby self-reactive T cells and B cells are eliminated through clonal deletion is an imperfect process, allowing some self-reactive lymphocytes to escape to the extra-thymic regions. Self-reactive lymphocytes are consequently present in the normal immune repertoire (500). The maintenance of peripheral tolerance by dendritic cells (DC), through cell-contact and cytokine mediated modulation of the immune response, is therefore critical in preventing pathological self-reactivity. Traditionally, tolerance has been considered a paradigm of maturation, whereby immature DC induce T cell anergy or Treg cells, but mature DC exclusively induce immunogenic primary T cell response. However, this is an over-simplistic model, particularly as processing and presentation of antigen, requires at least some DC maturation.

Evidence now indicates a role for semi-mature DC, loaded with tissue antigen, migrating to lymph nodes (501, 502). In the absence of additional 'danger' stimuli, such as Hsp60 or LPS, these DC are arrested in a semi-mature state and do not produce the pro-inflammatory stimuli required for T cell activation (503, 504). IL-10 and TGF- $\beta$  are classically associated with 'licensing' DC tolerogenicity by inhibiting DC maturation (505), although additional emerging tolerogenic inducing factors include vasoactive intestinal peptide (VIP), thymic stromal lymphopoeitin (TSLP) and G-CSF (506-509). Of note, self-antigen may be cross-presented to tolerising lymph node resident DC by short-lived DC that migrate through the afferent lymph from peripheral tissues (510). It is now generally accepted that DC induction of tolerance or immunogenicity relies on a combination of factors including DC lineage and maturation status (immature/semi-mature/mature), local cytokine milieu and antigen dose (Fig. 1.6). The expression of inhibitory receptors such as PDCD-1, DEC-205 and immunoglobulin-like transcript 3 and 4 (ILT3/ ILT4), as well as low NF- $\kappa$ B activation, are associated with semi-mature DC (511-513). However DC tolerogenicity is more a functional, as opposed to a lineage specific phenotype. Functional features include modulation of peripheral tolerance by

**Figure 1.6 Anergy, tolerance or activation is dependent upon dendritic cell maturation**

The current model of dendritic cell (DC) induction of anergy, tolerance or activation, whereby immature DC induct T cell anergy, but semi-mature DC can stimulate conversion of naïve T cells by a combination of co-stimulatory molecule expression and IL-10 release. Fully mature DC can promote T cell activation and clonal expansion, according to the traditional 2-signal model of T cell activation. However, in addition to this mature DC may also expand pre-existing Treg, which can in turn modulate the DC directed adaptive immune response.



expression of indoleamine 2, 3-dioxygenase (IDO); T cell anergy; induction/expansion of T regulatory (Treg) cells and production of IL-10.

#### **1.2.4.1 Indoleamine 2,3-dioxygenase**

Indoleamine 2,3-dioxygenase (IDO) mediates T cell homeostasis and self-tolerance by catabolism of the essential amino acid tryptophan to kynurenines in the local tissue environment which physiologically regulates T cell proliferation and survival (514). In chronic immune activation, tryptophan availability is reduced, leading to inhibition of cell proliferation. As such, IDO has a complex role in immunoregulation in infection, pregnancy, transplantation, neoplasia and autoimmunity. DC are distinct in their expression of IDO functional activity and in the modulation of this activity by IFN- $\gamma$ . This may represent a self-limiting feedback mechanism whereby autocrine or paracrine IFN- $\gamma$ , from Th1 and Treg cells, NK cells or NK T cells, induces DC IDO thus inhibiting T cell proliferation and triggering T cell apoptosis by depletion of tryptophan (515). Indeed, IFN- $\gamma$  ablates the disease-promoting potential of DC from NOD mice, and renders these cells protective in diabetic recipients (516). Furthermore, DC tolerogenic function can be restricted by CD40 ligation and IL-6 down-regulation of IFN- $\gamma$ R expression (517) and may represent a key mechanism of autoimmunity.

#### **1.2.4.2 DC induction of Treg**

Induction and expansion of CD4<sup>+</sup>/CD25<sup>+</sup>/Foxp3<sup>+</sup> Treg by semi-mature mDC and pDC triggered by treatment with tolerogenic ‘licensing’ factors, has been demonstrated in a number of models including G-CSF treatment in spontaneous type I diabetes in NOD mice (508, 509) and VIP differentiated DC transfer to murine EAE (507). The exact cellular mechanisms by which Treg are induced are not well defined. However, whether Treg are induced by pDC or mDC appears to be a function of TLR stimulation, and as such may indicate a pivotal collaborative point whereby one DC subset provides regulation of the immune response induced by the other.

While DC function is undoubtedly critical to maintenance of the immune system it may also carry inherent immunological risks. It has been hypothesised that maturing DC may present peptides from dying self-tissue and environmental proteins as well as from pathogens, when encountered in a highly inflamed environment. While immature DC

induced antigen-specific peripheral tolerance in the steady state should account for this risk, in susceptible individuals chronic inflammatory and autoimmune disease may ensue.

### **1.3 Dendritic Cells and Autoimmune Disease**

Autoimmune disorders are characterised by the abolition of self-tolerance and subsequent emergence of self-reactive lymphocytes. As the only professional APC recognised to date as capable of provoking an autoimmune disease, DC have been implicated in the initiation and perpetuation of systemic lupus erythematosus (SLE) (518), experimental autoimmune encephelomyelitis (EAE) (519), thyroiditis (520), diabetes (521), Sjögrens Syndrome (522), multiple sclerosis (523), psoriasis (524), psoriatic arthritis (525), juvenile idiopathic arthritis and rheumatoid arthritis (273). Numerous animal models of spontaneous autoimmune disease have identified DC as amongst the first cells to infiltrate target tissue, highlighting the potency with which DC, expressing endogenous self-peptides or pulsed *ex vivo* with immunogenic self-peptide, can prime autoreactive T cells (526) (527-529).

#### **1.3.1 Evidence for a Role for Dendritic Cells in Inflammatory Arthritis**

##### **1.3.1.3 Dendritic Cells in Inflamed Synovium**

The arthritic synovium, considered an ectopic lymphoid organ, is characterised by a complex cellular infiltrate composed of T cells, B cells, monocytes, macrophages, mast cells and DC (295). DC are enriched in both synovial fluid (SF) (530-532) and synovial membrane (SM) (274, 533-535). SF DC, despite the inflammatory environment, express a less mature, CD80<sup>-</sup>/CD83<sup>lo</sup> phenotype most likely resulting from the presence of suppressive factors such as TGF- $\beta$ , which has been shown to inhibit DC-T cell interactions in patients with chronic arthritis (536, 537). Conversely, SM DC are found in the perivascular area, localised to lymphocytic aggregates and are reported to exhibit a predominantly mature phenotype with high expression of T cell costimulatory molecules, MHC I and II (535, 538), nuclear RelB (539), adhesion molecules and STAT 4 (540). Immunohistochemical analysis of SM chemokine expression has correlated this mature CCR7<sup>+</sup> DC homing to lymphocyte derived CCL19/21 release (534). The DC maturation gradient may reflect migration of immature SF DC to the SM and *in situ* maturation, supported by the localisation of immature CCR6<sup>+</sup>/CD1a<sup>+</sup> DC in macrophage inflammatory

protein (MIP)-3 $\alpha$  (CCL-20) rich areas of the synovial lining layer (534, 541). Alternative evidence supports a model whereby over-expression of the precursor DC chemoattractants MIP-1 $\alpha$ , MCP-1 and RANTES, coupled with the TNF- $\alpha$  driven up-regulation of adhesion molecules induces the transendothelial migration of PB DC direct to the SM (538). According to this model, monocyte shedding to the SF and subsequent differentiation into mDC may account for SF DC, supported by the presence of multiple growth and differentiation factors including GM-CSF, TNF- $\alpha$  and IL-1 (538, 542). However, this does not account for the presence of immature pDC in SF (106, 125).

Clearly, the analysis of DC infiltrates in the inflamed synovium is by no means definitive with the complexity inherent in studying such a transient population of cells amplified by a paucity of specific DC markers and reliable quantitation. While mDC are enriched in both SF and SM, pDC have been only sparsely analysed but are also found in SF and SM (106, 125). The relatively recent identification of the novel pDC marker CD303 (BDCA-2) (397) will facilitate the more thorough characterisation of pDC and their role, if any, in disease pathogenesis. Of interest, numerous cases of arthritis triggered by IFN- $\alpha$  therapy of previously non-arthritic patients, which resolved following cessation of treatment, have been reported (543-549), implicating this type I interferon in disease pathology and, by association, the cells which produce it. Thus the necessity for more thorough characterisation of the presence and functional role of different DC subsets in inflammatory arthritis is clear. It can be envisaged that greater understanding of the involvement of the innate events involved in disease pathology could lead to therapeutic strategies inhibiting aberrant perpetual activation

Clearly, extensive evidence from human studies implicates DC in the pathogenesis of inflammatory arthritis. However, elucidation of the direct functional roles that DC play in the initiation and perpetuation of the disease has come from exemplary murine studies.

#### **1.3.1.2 DC involvement in murine models of arthritis**

The early infiltration of (m) DC to the target tissue of inflammation in collagen-induced arthritis (CIA), spontaneous arthritis in MRL/*lpr* mice (550) and antigen-induced arthritis in rats (551, 552) has been demonstrated. Of particular note, Brewer and Leung have clearly demonstrated the capacity for DC in arthritis induction using *ex vivo* collagen type

II-pulsed, bone-marrow differentiated mDC, to induce arthritis in joints adjacent to the site of injection (553). Furthermore, administration of TNF antagonists was sufficient to inhibit arthritic progression in this model. Similarly, adoptive transfer of T-box expressed in T cells (T-bet) expressing DC was sufficient to re-constitute inflammation in RAG2<sup>-/-</sup> Tbet<sup>-/-</sup> mice, otherwise resistant to collagen antibody-induced arthritis (554). Of particular interest, transfer of DC, genetically modified to over-express IL-4, to murine with established CIA reduced IFN- $\gamma$  production and collagen type II reactivity thus reducing disease severity and demonstrating a potential for manipulation of DC as a therapeutic vector (555). To date, no analysis of the potential contribution of pDC to murine models of arthritis has, as yet, been undertaken.

### **1.3.1.3 Pharmacological modification of DC**

Given the central role that DC play in the maintenance of immunity and induction of tolerance, in combination with the evidence supporting their integral functional role in initiating inflammation, they present an attractive therapeutic target. However, much of the evidence – particularly from murine models – concerns their role in the triggering stages of arthritis. The majority of therapies are aimed at disrupting the mechanisms perpetuating inflammation, as it is at this stage that most patients are diagnosed with disease. While a number of drugs have biological effects on DC, the link between these effects and the therapeutic efficacy are unclear (Table 1.8). Promisingly, a number of studies analysing the efficacy of a variety of therapies, including disease modifying anti-rheumatic drugs (DMARDs), TNF-blocking therapies and the newer CTLA4Ig have observed specific effects on DC, which may be intrinsically associated with the therapeutic efficacy of these drugs.

#### **1.3.1.3.1 DMARDs and DC**

The active metabolite of the potent DMARD, leflunomide, A77 1726, interferes with mDC function by reducing expression of the co-stimulatory molecules CD40 and CD80 and subsequently abrogating DC initiated T cell proliferation. Furthermore, both IL-12p70 and TNF- $\alpha$  production is impaired and NF $\kappa$ B completely suppressed by A77 1726 and its derivative FK778, even in the presence of LPS or CD40L (556, 557).

**Table 1.8** Biological effects of drugs on DC

Agent	Biological Action on DC
Corticosteroids	Suppresses number of pDC precursors; restrains monocyte differentiation into DC; suppresses IL-12 production; enhances IL-10 production by DC(558-560)
1,25 (OH) <sub>2</sub> vitamin D <sub>3</sub>	Restrains monocyte differentiation into DC; suppresses monocyte derived DC maturation; suppresses IL-12 production; enhances IL-10 production by DCs; modulates DC responses to chemotactic signals(561, 562)
Hydrochloroquine	Prevents lysosomal vacuole acidification(563)
Cyclosporin	Suppresses TNF production(564)
Mycophenolate	Retards DC maturation; reduces IL-12 production(565)
CD2 binding fusion protein (Alefacept)	Reduces CD83 <sup>+</sup> and CD11c <sup>+</sup> expressing DC in psoriatic lesions in psoriasis ; suppresses IL-23 production(566)
Anti-CD11a (Efalizumab)	Reduced infiltration of langerin <sup>+</sup> /CD14 <sup>+</sup> /HLA-DR <sup>+</sup> /CD40 <sup>+</sup> /CD86 <sup>+</sup> /DC-LAMP <sup>+</sup> /CD83 <sup>+</sup> DC to psoriatic skin lesions in psoriasis(567)
A77 1726, the active metabolite of Leflunomide	Suppresses IL-12 production by mDC; reduces expression of co-stimulatory molecules; inhibits NFκB, suppresses TNF-α production by mDC(556, 557)
CTLA-4Ig	Activates pDC IDO production(568)



#### ***1.3.1.3.2 TNF neutralisation and DC***

Analysis of the mode of action of TNF blocking (etanercept) therapy has been assessed in psoriasis. Immunohistochemical analyses revealed that TNF blocking led to a gradual decrease in CD11c<sup>+</sup> DC infiltration to psoriatic plaques, which was likely attributable to a decrease in MIP-3 $\alpha$ . Also decreased was both IL-23 mRNA and iNOS mRNA and protein commensurate with a decrease in DC activity (569). Consistent with this report, TNF- $\alpha$  has been shown to enhance the phenotypic and functional maturation of human Langerhans cells, with up-regulation of CD40/CD54/CD86/CD83/DC-LAMP/CCR7 and down-regulation of langerin, inducing both IL-12p40 and IFN- $\gamma$ -inducible-protein (IP-10)/CXCL-10, a Th1-attracting chemokine (570). Therefore TNF blockade may break the potentially self-sustaining cycle of DC activation and maturation.

#### ***1.1.3.1.3 CTLA-4Ig and DC***

CTLA-4Ig binds CD80/86 with high affinity, thus blocking CD28 and inhibiting DC-T cell contact and T cell activation. CTLA-4 Ig infusion has been shown to reverse the activation status of DC in psoriatic plaques as well as their accumulation to psoriatic plaques. (571). Furthermore, the study suggested that the specific co-stimulation of T cells through CD28 is pivotal to creating the inflamed microenvironment in which autoimmune inflammation is perpetuated. However, these signals alone are not sufficient to initiate the chronic inflammatory cascade – this is likely to occur due to a number of factors including genetic pre-disposition of the host, increased levels of autoantigen and autoreactive T cells, elevated MHC II antigens and enhanced inflammatory cytokines. In this study B7-CD28/CTLA4 blockade down-regulated a variety of pro-inflammatory stimuli resulting in a longer clinical response than had been observed in other T-cell based therapies. The durable response may have been attributable to IFN- $\gamma$  secretion, triggered by CTLA-4 Ig. IFN- $\gamma$  has an immediate autocrine effect upon DC that results in IDO secretion (568), which was up-regulated in psoriatic plaque infiltrating pDC (discussed *1.2.4.1*). Of note, CTLA-4Ig was not toxic to DC, thus their depletion in the psoriatic lesions was probably attributable to down-regulation of endothelial adhesion molecules thus impairing their ability to enter the site.

## 1.4 Summary and Hypothesis

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are chronic inflammatory arthropathies of heterogeneous aetiology affecting 2-3% of the general population. Extensive *in vivo* and *in vitro* studies have identified the multiple pro-inflammatory cytokines and enzymes implicated in the pathogenesis of these disorders culminating in therapeutically beneficial cytokine targeted therapies. Cytokine blockade however exhibits variable responses across patient populations and importantly disease activity recurs upon cessation of therapy. The critical challenge in therapeutics now is to uncover the underlying immunological processes responsible for the generation and perpetuation of inflammatory arthritis such that peripheral tolerance can be re-established and therapeutic remission can be maintained. Of particular interest are the co-stimulatory molecule and cytokine mediated interactions bridging the innate and adaptive immune response, notably those initiated by the interaction of dendritic cells (DC) with T cells, as the evolution of the latter is profoundly influenced by these cognate and soluble interactions. DC are the only APC recognized to date as capable of provoking autoimmune disease. DC biology thus embodies an important line of investigation in determining the initial triggers that generate and (of therapeutic significance) maintain chronic inflammatory arthritis. However, to date there have been very few investigations of the phenotype and function of DC, in particular with reference to DC lineage.

The central hypothesis of this thesis is that the comprehensive characterisation of dendritic cell subsets, plasmacytoid (p) DC and myeloid (m) DC, in the blood and primary target tissue of chronic inflammatory arthritis patients would clarify their contribution to the initiation, modulation and perpetuation of inflammatory arthritis. Accordingly, this thesis has utilized a combination of *in vitro* and *in vivo* analyses in order to examine the aberrant accumulation to, and function within, the inflamed synovium from PsA and RA patients.

### 1.4.1 Specific Aims and Objectives

- Characterise mDC and pDC phenotype and provisional function in inflammatory synovitis patients;
- Explore the effects of TNF blockade on such DC subsets;

- Perform *in vitro* assays to determine human DC function *ex vivo*;
- Explore the regulation of DC subsets *in vitro* using a novel cell culture model – in particular to rationalise the co-expression of TNF and IL-15 in this context; and
- Use an *in vivo* model of inflammatory arthritis to formally test the hypothesis that DC subsets perform distinct roles in initiation and perpetuation of inflammatory arthritis.

## **Chapter 2: Materials and Methods**

## **2.1 Patients and Controls**

Inflammatory arthritis patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (23), or met a diagnostic criteria for PsA as previously described (572, 573). All patients and healthy donors gave informed consent and the study protocol was approved by the Ethical Committee, Glasgow Royal Infirmary, Scotland, and by the Medical Ethics Committee of the Academic Medical Center in Amsterdam, The Netherlands. Specific patient details for each study are located in the pertinent results chapters (3.2.1, 4.2.1, 5.2.1).

### **2.1.1 Clinical assessment**

Full demographic details and list of current medications were recorded for each visit at the time that sample (peripheral blood, synovial fluid or synovial membrane) was obtained.

### **2.1.2 Parameters of disease activity**

Disease activity was assessed during clinic visit by the clinician using standard hospital diagnostic laboratory techniques.

#### ***2.1.2.1 Joint scores***

Clinical assessment was performed at the screening visit for tenderness and swelling of each peripheral joint. The Disease Activity Score in 28 joints (DAS28) was calculated.

#### ***2.1.2.2 C-reactive protein***

C-reactive protein (CRP) levels were measured by standard nephelometry and are reported in units of mg/L.

#### ***2.1.2.3 Erythrocyte sedimentation rate***

Erythrocyte sedimentation rate (ESR) was measured by standard Westergren method and are reported in units of mm/hour.

#### ***2.1.2.4 Auto-antibodies: rheumatoid factor***

Rheumatoid factor (RF) was measured by Enzyme-Linked-ImmunoSorbent Assay (ELISA).

## 2.2 Animals

6-8 week old female BALB/c mice were purchased from Harlan (Bicester, UK) and used for *in vitro* experiments and as Tg CD4<sup>+</sup>/KJ1.26<sup>+</sup> T cell transfer recipients in the OVA TcR transgenic model of arthritis. DO11.10 BALB/c TCR Tg mice containing CD4<sup>+</sup>T cells expressing a TCR that recognizes the chicken OVA peptide 323-339 complexed with the MHC class II molecule I-Ad (detected by the clonotypic mAb KJ1.26) (574), were used as Tg CD4 T cell donors (bred in-house, Central Research Facility, University of Glasgow, Glasgow, UK). 6-8 week old female DBA/1 mice were purchased from Harlan (Bicester, UK) and used for *in vitro* experiments and in the collagen-induced arthritis (CIA) model.

## 2.3 Antibody Preparation

### 2.3.1 F(ab')<sub>2</sub> fragmentation

Human anti-IL-15 and human IgG (both were supplied by Genmab, Amsterdam) were fragmented using an Immunopure® F(ab')<sub>2</sub> Preparation Kit according to the manufacturer's instructions (Pierce). Briefly, 1.0 mg of antibody was added to a tube containing immobilized pepsin and incubated for 4 hours in a shaking water bath at 37°C. A resin separator was used to separate digest from the immobilized pepsin, and 1.5 ml of Immunopure® IgG Binding Buffer was added. The digest was then added to an equilibrated column and throughput collected. The fragmented antibody was dialyzed at 50K MWCO, to remove small Fc fragments.

### 2.3.2 Antibody purification

The pDC depleting antibody Rat anti-mouse 120G8 IgG<sub>1κ</sub> was a generous gift from Dr James Brewer, University of Strathclyde, UK. The isotype control YCATE.55.9.1 rat anti-dog CD8 IgG<sub>1κ</sub> was a generous gift from Mark Frewin (Sir William Dunn School of Pathology, University of Oxford, UK). Antibody was purified from culture ascites using the Montage® Antibody Purification<sub>PROSEP®</sub> Kit (Millipore) according to the manufacturer's instructions. Briefly, samples were diluted 1:1 in binding buffer (1.5 M glycine/NaOH buffer, 3 M NaCl, pH 9.0) and added to an equilibrated spin column and centrifuged at 100-150 g for 20 minutes. The throughput was collected and added back to the spin column as above and repeated 4 times as Rat IgG<sub>1</sub> has low affinity binding. Spin columns were washed in binding buffer by 2 minute centrifugation at 500 g. The bound

IgG was then eluted by addition of 10 ml elution buffer (0.2 M glycine/HCl buffer pH 2.5) and centrifugation for 5 minutes at 500 g into a tube containing 1.3 ml of neutralization buffer (1 M Tris/HCl buffer pH 9.0). The antibody preparation was concentrated by centrifugation through an Amicon Ultra-15 centrifugal filter device, 30,000 NMWL (Millipore). Dialysis tubing was prepared by boiling for 5 minutes (Medicell International Ltd). Concentrated antibody preparations were de-salted by dialysis into PBS overnight at 4°C.

### **2.3.3 Protein Concentration Assay**

The final antibody concentration was determined using a BCA<sup>TM</sup> Protein Assay Kit according to the manufacturer's instructions (Pierce). Briefly, 25 µl of each BSA standard or unknown sample was added in triplicate to a 96 well NUNC MaxiSorp microplate followed by 200 µl of BCA<sup>TM</sup> working reagent. Plates were incubated at 37°C for 30 minutes and then absorbance measured at 570 nm. The concentration of antibody in solution was proportional to the optical density (OD) of the standards, and concentrations were calculated using Microsoft Excel.

## **2.4 Cell culture**

All cell culture reagents were purchased from Invitrogen Life Technologies, Sigma-Aldrich or Gibco unless otherwise stated (see 2.10 for supplier's addresses). All cells were cultured at 37°C 5% CO<sub>2</sub> in RPMI-1640 complete medium containing L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin B (1.25 µg/ml) and 10% foetal calf serum (FCS) (all Sigma-Aldrich). For human pDC and mDC cultures, complete medium did not contain 10% FCS but was instead supplemented with 5% human AB serum (Sigma-Aldrich).

### **2.4.1 Cell Lines**

#### **2.4.1.1 BDB2**

BDB2 cells, an IL-15-dependent, human T cell line, were a kind gift of Dr. John Campbell, University of Glasgow, UK. BDB2 cells were cultured in complete medium supplemented with 10 ng/ml IL-15 (R&D Systems).

#### **2.4.1.2 X63**

X63 cells are a GM-CSF transfected myeloma cell line and were a kind gift of Brigitta Stockinger, National Institution for Medical Research, London, UK. X63 cells were cultured in complete medium and the culture ascite used to differentiate murine bone marrow cells into myeloid dendritic cells.

#### **2.4.1.3 DO11.10 GFP T cell hybridoma**

The DO11.10 GFP T cell hybridoma is a cell line that responds to presentation of OVA peptide (323-339) on the MHC Class II molecule (I-A<sup>d</sup>) and were a kind gift from Dr James Brewer, University of Strathclyde, UK. Cells were cultured for 24 hours before adding 100 µl/10ml Geneticin® (G418) (GIBCO, BRL) and 200 µl/10ml HEPES buffer (Sigma).

### **2.4.2 Primary human cell culture**

#### **2.4.2.1 Purification of mononuclear cells from synovial fluid or peripheral blood**

Synovial fluid (SF) or peripheral blood (PB) mononuclear cells (MC) were purified by standard density gradient centrifugation. Briefly, SF or PB was obtained by needle effusion of the swollen joint or venepuncture to heparinised tubes (Leo Laboratories Ltd). SF or PB was then diluted 1:1 in phosphate buffered saline (PBS) (Gibco) and overlaid on Histopaque®-1077 (Sigma) at 3:1 in 15 ml centrifuge tubes. Tubes were centrifuged at 800 g for 20 minutes at room temperature. The mononuclear cell layer was then harvested from each tube and washed three times at 300 g for 10 minutes in PBS, prior to use for cell culture, or ice cold PBS containing 2mM ethylene diamine tetra-acetic acid (EDTA) and 0.5% bovine serum albumin (BSA), for cell purification (both Sigma).

#### **2.4.2.2 Isolation of myeloid and plasmacytoid dendritic cells**

Plasmacytoid dendritic cells (pDC) and myeloid dendritic cells (mDC) were sequentially purified by magnetic cell sorting (MACS; Miltenyi Biotec) using a high gradient Mini-MACS® device, according to the manufacturer's instructions. Briefly, in the experiments described in chapter 3, mononuclear cells (2.4.2.1) were resuspended in 200 µl PBS/0.5% BSA/2mM EDTA containing 100 µl/10<sup>7</sup> cells anti-CD304 (BDCA-4) magnetic microbeads and 100 µl FcR block (both Miltenyi Biotec) for 18 minutes at 4°C, followed by centrifugation at 300 g for 10 minutes. Cells were then resuspended in 500 µl



PBS/0.5%BSA/2mM EDTA buffer and CD304 (BDCA-4)<sup>+</sup> (pDC) purified by positive selection over a pre-equilibrated magnetic MS column (Miltenyi Biotec). Unlabelled cells were collected and, after washing by centrifugation at 300 g for 10 minutes, resuspended in 200  $\mu$ l PBS/0.5%BSA/2mM EDTA containing 100  $\mu$ l anti-CD19-conjugated magnetic microbeads (for B cell depletion) and 100  $\mu$ l of FcR block and 100  $\mu$ l of biotin-conjugated anti-CD1c for 18 minutes at 4°C (all Miltenyi Biotec), followed by centrifugation at 300 g for 10 minutes. B cells were then depleted by retention in a magnetic MS column as described above. The throughput was collected, and after centrifugation at 300 g for 10 minutes, resuspended in 200  $\mu$ l PBS/0.5% BSA/2mM EDTA containing 100  $\mu$ l/10<sup>7</sup> cells anti-biotin-conjugated magnetic microbeads (Miltenyi Biotec) for 18 minutes at 4°C, followed by centrifugation at 300 g for 10 minutes. CD1c<sup>+</sup>mDC were then isolated by positive selection over a pre-equilibrated magnetic MS column.

For the experiments described in chapter 5, CD19<sup>+</sup> B cells were first depleted from mononuclear cells followed by CD1c<sup>+</sup> mDC purification as described above. pDC were then isolated by negative selection by incubating the remaining cells in monoclonal biotin conjugated antibodies against antigens not expressed by pDC. Thus T cells, B cells, NK cells, mDC, monocytes, granulocytes and erythroid cells were labelled by 10 minute incubation at 4°C in 100  $\mu$ l of biotin-conjugated non-pDC antibody cocktail (Miltenyi Biotec), followed by two washes at 300 g for 10 minutes and incubation with 100  $\mu$ l anti-biotin-conjugated magnetic microbeads for 15 minutes at 4°C. Non-pDC cells were then removed by retention in a magnetic LS column (Miltenyi Biotec). Unlabelled cells in the throughput were therefore pDC. Purity and phenotype of pDC and mDC was assessed by flow cytometry (as described in 2.6.2) and routinely >95% for PB purifications and >93% for SF purifications (<10% neutrophil contamination).

#### ***2.4.2.3 Isolation of CD3<sup>+</sup> T cells and CD14<sup>+</sup> Monocytes***

CD3<sup>+</sup> T cells and CD14<sup>+</sup> monocytes were purified by positive selection from mononuclear cell suspension by MACS, according to the manufacturer's instructions. Briefly, mononuclear cells were incubated in 200  $\mu$ l PBS/0.5% BSA/2mM EDTA containing 100  $\mu$ l/10<sup>7</sup> cells anti-CD3-conjugated magnetic microbeads, or 100  $\mu$ l/10<sup>7</sup> cells anti-CD14-conjugated magnetic microbeads (both Miltenyi Biotec) for 18 minutes at 4°C. After washing in PBS/0.5% BSA/2mM EDTA by centrifugation at 300 g for 10 minutes, CD3<sup>+</sup> T cells or CD14<sup>+</sup> monocytes were isolated by positive selection over a pre-equilibrated MS

column (Miltenyi Biotec). Purity was assessed by flow cytometry (2.6.2) and routinely >98%.

#### **2.4.2.4 mDC and pDC stimulation**

To ascertain function of DC subsets, stimulation using TLR agonists was used. Isolated mDC (2.4.2.2) were incubated in triplicate at  $1-5 \times 10^5$ /ml containing 50 ng/ml GM-CSF (StemCell Technologies) alone or supplemented with the TLR2 agonist *Staphylococcus aureus* peptidoglycan (PGN) at 10 µg/ml or the irrelevant control, TLR9 agonist CpG oligodeoxynucleotide (ODN) 2216 (ggGGTCAAGCTTGAgggggG) at 3.2 µg/ml (both InVivogen). Isolated pDC (2.4.2.2) were incubated in triplicate at  $1-5 \times 10^5$ /ml containing 20 ng/ml IL-3 (StemCell Technologies) alone or supplemented with CpG ODN 2216 at 3.2 µg/ml or ODN 2216c (control for CpG ODN 2216) (ggGGGAGCATGCTCgggggG) at 3.2 µg/ml (both InVivogen). After 24 hours at 37°C 5% CO<sub>2</sub>, cultures were centrifuged, supernatants collected and stored at -20°C until analysis by multiplex (2.7.2) and phenotypical analysis of mDC and pDC was performed by flow cytometry methods (2.6.2).

#### **2.4.2.5 mDC/T cell contact and pDC/T cell contact assays**

CD3<sup>+</sup> T cells were purified by positive selection (2.4.2.3) from PB and blasted by 48 hour culture in complete medium containing 5 µg/ml phytohaemagglutinin (PHA), in order to produce an activated phenotype similar to that displayed in synovium. At 48 hours, mDC and pDC were sequentially purified by positive (mDC) and negative (pDC) selection from autologous PB donors (2.4.2.2). Isolated mDC were plated in flat-bottom 96 well plates in triplicate aliquots at  $0.5-1 \times 10^5$ /well in complete media containing 50 ng/ml GM-CSF (StemCell Technologies). Isolated pDC were plated in flat-bottom 96 well plates in triplicate aliquots at  $0.2-0.8 \times 10^5$ /well containing 20 ng/ml IL-3 (StemCell Technologies). The blasted T cells were either fixed in PBS/2% paraformaldehyde on ice for 2 hours, or not (as indicated), and added back to mDC and pDC cultures at a ratio of 3:1. T cells were separated from mDC and pDC by cell membrane inserts where indicated (Nunc). After 24 hours of incubation at 37°C 5% CO<sub>2</sub>, supernatants were harvested and cytokines measured via multiplex analysis (2.7.2).

#### ***2.4.2.6 Differentiation of monocyte derived dendritic cells***

CD14<sup>+</sup> monocytes were purified from PBMC by positive selection (2.4.2.3) and cultured in 12 well plates at  $2 \times 10^6$  cells/well in complete medium supplemented with 20 ng/ml IL-4 and 50 ng/ml GM-CSF (both StemCell Technologies), or by addition of 10% cell free osteoarthritis (OA), systemic-lupus erythematosus (SLE), psoriatic arthritis (PsA) or rheumatoid arthritis (RA) synovial fluid (SF) for 5 days. The cell free SF from each diagnostic group was composed of a cocktail of SF taken from 5 separate patients. Monocyte-derived DC (moDC) phenotype was assessed by flow cytometry analysis of surface CD1c and CD11c and maturation marker expression (2.6.2).

### **2.4.3 Murine cell culture**

#### ***2.4.3.1 mDC generation from bone marrow***

Bone marrow was isolated from mouse femurs and a cell suspension prepared and filtered through Nytex to minimize clumps. To generate mDC, bone marrow cells were cultured at  $5 \times 10^5$  cells/well, in a 6 well culture plate (Corning Costar), in complete medium supplemented with 10% conditioned medium generated by the GM-CSF-transfected X63 myeloma cell line for 6 days at 37°C/5%CO<sub>2</sub>. On day 6, immature mDC were harvested by gentle scraping and used as a source of DC.

#### ***2.4.3.2 pDC generation from bone marrow and purification***

Bone marrow was isolated as described above. To generate pDC, bone marrow cells were cultured at  $2 \times 10^6$ /well, in a 6 well culture plate (Corning costar), in complete medium supplemented with 100ng/ml human Flt-3L (generous gift from Professor A Mowat, University of Glasgow, UK) for 8 days at 37°C 5% CO<sub>2</sub>. pDC were then purified by positive magnetic cell sorting (MACS, Miltenyi Biotec), according to the manufacturer's instructions. Briefly, cells were harvested by gentle scraping and then incubated in 200 µl PBS/0.5% BSA/2mM EDTA containing 100 µl anti-mouse plasmacytoid dendritic cell antigen (mPDCA)-1-conjugated magnetic microbeads for 18 minutes at 4°C (Miltenyi Biotec). After washing in PBS/0.5% BSA/2mM EDTA by centrifugation at 300 g for 10 minutes, mPDCA-1<sup>+</sup> pDC were isolated by positive selection over a pre-equilibrated MS column. mPDCA-1<sup>+</sup> pDC were harvested from one column and immediately added to a fresh pre-equilibrated column to enhance purity. Purity was assessed by flow cytometry and routinely >95% (2.6.4).

#### ***2.4.3.3 Isolation of CD4<sup>+</sup> T cells from lymph nodes and spleen***

Lymph nodes were harvested from DBA-1 mice undergoing collagen-induced arthritis (CIA), and lymph nodes and spleen were harvested from naïve DBA-1 mice. Single cell suspensions were prepared from spleen by crushing under Nytex (Cadisch Precision Meshes), followed by red cell lysis in 5 ml water for injection for 10 seconds, rapidly followed by addition of 5 ml of two times concentrated PBS. Single cell suspension was prepared from lymph nodes by crushing under Nytex. After washing, all cells were pushed through nytex to minimise clumping. CD4<sup>+</sup> T cells were purified by positive magnetic cell sorting (MACS, Miltenyi Biotec), according to the manufacturer's instructions. Briefly, cells were incubated in 200 µl PBS/0.5% BSA/2mM EDTA containing 100 µl anti-CD4-(L3T4) conjugated magnetic microbeads for 18 minutes at 4°C (Miltenyi Biotec). After washing in PBS/0.5% BSA/2mM EDTA by centrifugation at 300 g for 10 minutes, CD4<sup>+</sup> T cells were isolated by positive selection over a pre-equilibrated MS column. Purity was assessed by flow cytometry (2.6.5) and routinely >98%.

#### ***2.4.3.4 Antigen-specific proliferation by mDC/ T cell and pDC/T cell co-culture***

Antigen-specific responses were assessed by tritiated thymidine uptake proliferation assay to evaluate DNA synthesis. Bone-marrow differentiated mDC (2.4.3.1) and pDC (2.4.3.2) were cultured in triplicate in complete medium at  $0.5 \times 10^5$ /well, in a flat-bottomed 96 well culture plate (Corning Costar). Cells were cultured as indicated with or without 50 µg/ml bovine tracheal collagen type II (CII; Sigma-Aldrich), for at least 6 hours. To some of the pDC cultures was added 3.2 µg/ml CpG ODN 1826 (mouse) (TCCATGACGTTTCCTGACGTT) (InVivogen) 2 hours prior to addition of CD4<sup>+</sup>T cells. CD4<sup>+</sup> T cells were purified from DBA/1 mice, either naïve or undergoing collagen-induced arthritis (2.4.3.3) and added to the DC cultures at a ratio of 3:1. At 48 hours 0.5 µCi/well [6-<sup>3</sup>H]-thymidine (Amersham Pharmacia Biotech) was added to each well and allowed to incorporate into cellular DNA for 8-12 hours. Cells were harvested onto glass fibre filter mats (Perkin Elmer) using a Betaplate 96-well harvester (Amersham Pharmacia Biotech). Incorporated label was assessed by liquid scintillation counting and results are expressed as the Index of Stimulation, calculated as the proliferation in triplicate wells supplemented with CII divided by the proliferation in matched triplicate cultures in media alone.

#### ***2.4.3.5 mDC and pDC presentation of OVA peptide to DO11.10 GFP transgenic T cells***

The DO11.10 GFP T cell hybridoma is a cell line that responds to presentation of OVA peptide (323-339) on the MHC Class II molecule (I-A<sup>d</sup>). When the T cell hybridoma receives this stimulation, it induces transcription of Nuclear Factor of activation of T cells (NFAT). Modification of the hybridoma by the addition of a NFAT reporter construct means that when NFAT is produced, it binds to a NFAT specific binding site, which regulates a green fluorescent protein (GFP) inducing promoter (575). In essence, when the T cell hybridoma sees MHC/peptide complex, it fluoresces. Bone marrow differentiated mDC (2.4.3.1) at day 5 of culture, and pDC (2.4.3.2) at day 8 of culture, were harvested by gentle scraping and then plated into a 24 well plate (Greiner) in 1 ml complete medium at  $5 \times 10^5$  cells/ml after washing by centrifugation at 300 g for 5 minutes. After 24 hours  $5 \text{ mg/ml}$  OVA<sub>323-339</sub> (Worthington) was added to half of the wells. After 6 hours  $5 \times 10^5$  DO11.10 GFP T cell hybridoma was added to all wells and cultures incubated overnight at 37°C 5% CO<sub>2</sub> (v/v). Non-adherent cells were then harvested from wells, and washed in PBS at 300 g for 5 minutes. The cells were resuspended in PBS/0.5%BSA/0.1%NaN<sub>3</sub> and counter-stained in CD4 (L3T4)-PE or Rat IgG<sub>2a</sub>-PE isotype control (both R&D Systems) for 20 minutes on ice. Cells were then washed three times in PBS/0.5% BSA/0.1% NaN<sub>3</sub>, resuspended in PBS and fluorescence measured by flow cytometry on a FACScalibur (Becton Dickinson).

## **2.5 Murine models of arthritis**

### **2.5.1 Collagen-Induced Arthritis**

Collagen-induced arthritis (CIA) was elicited in 6-8 week old DBA/1 mice (2.2). Mice were immunized by intradermal injection of 200 µg of acidified bovine type II collagen (CII) (Chondrex Inc) emulsified in Complete Freund's Adjuvant (CFA) (BD Biosciences). Mice were boosted intra-peritoneal (i.p) with CII (200 µg in PBS) on day 21. Mice were monitored for signs of arthritis based on erythema, swelling or loss of function present in each paw on a scale of 0–3, giving a maximum score of 12 per mouse. After onset of arthritis, mice were sacrificed and lymph nodes harvested for in vitro analysis (2.4.3). CIA was administered by Dr Michelle Ierna (MD Biosciences, UK).

## **2.5.2 Induction of experimental arthritis by breaking self-tolerance**

### ***2.5.2.1 Preparation of cell suspension for adoptive transfer***

This system produces a peptide/MHC-specific DO11.10 TCR-transgenic T cell population within a syngeneic recipient. The T cells from DO11.10 mice contain rearranged TCR-V $\alpha$  and TCR-V $\beta$  genes that encode a TCR specific for the chicken ovalbumin (OVA) peptide 323-339 (OVA<sub>323-339</sub>) bound to I-A<sup>d</sup> MHC class II molecules (574). These cells can be detected by flow cytometry using the clonotypic KJ1.26 monoclonal antibody (mAb) conjugated to fluorescent fluorochrome (576). Since KJ1.26<sup>+</sup> T cells are not produced in BALB/c at detectable levels, BALB/c are ideal transfer recipients allowing tracking of the transferred cells (577).

### ***2.5.2.2 Purification of CD4<sup>+</sup> T cells from DO11.10 BALB/c***

Axillary, brachial, inguinal, cervical, mesenteric and popliteal lymph nodes from DO11.10 BALB/c mice were pooled and single cell suspensions prepared by forcing through Nytex (Cadisch Precision Meshes) with a syringe plunger. Cells were washed in complete medium at 400 g for 5 minutes. Cell pellets were resuspended in PBS/0.5% BSA/2 mM EDTA containing anti-CD19, anti-CD11c, anti-CD16 and anti-CD8 mAbs (Serotec) and incubated for 18 minutes at 4°C. After washing by centrifugation at 300 g for 10 minutes, anti-IgG magnetic beads (Miltenyi Biotec) were added to cell suspensions and binding allowed to take place for 18 minutes at 4°C. B cells, monocytes, NK cells and CD8<sup>+</sup>T cells were therefore depleted by positive selection over LS columns (Miltenyi Biotec). The flow through was collected and contained only CD4<sup>+</sup> T cells. The % KJ1.26<sup>+</sup>/CD4<sup>+</sup> T cells in cell suspension was determined by flow cytometry methods. Briefly, cells were resuspended in PBS/0.5% BSA/0.1% NaN<sub>3</sub> and KJ1.26-FITC (BD Pharmingen) and counter-stained in CD4 (L3T4)-PE or Rat IgG<sub>2a</sub>-PE isotype control (both BD Biosciences) for 20 minutes on ice. Cells were then washed three times in PBS/0.5% BSA/0.1% NaN<sub>3</sub>, resuspended in PBS and fluorescence measured by flow cytometry on a FACScalibur (Becton Dickinson).

### ***2.5.2.3 Th1 differentiation of CD4<sup>+</sup> T cells***

Spleens were taken from DO11.10 BALB/c and single cell suspensions prepared and treated with mitomycin C (Sigma-Aldrich). Th1 differentiation of T cells was induced by culturing at  $2 \times 10^5$ /ml CD4<sup>+</sup> T cells with  $2 \times 10^6$ /ml mitomycin C treated APC and 0.3  $\mu$ M

OVA<sub>323-339</sub> (Genosys) in complete medium supplemented with 5 ng/ml IL-12 (PeproTech EC Ltd) and 10 µg/ml anti-IL-4 mAb (R&D Systems). After 3 days of culture, cells were harvested and washed by centrifugation at 400 g for 5 minutes. The % KJ1.26<sup>+</sup>/CD4<sup>+</sup> T cells in cell suspension and intracellular IFN-γ (to confirm Th1) was determined by flow cytometry methods (2.6.5).

#### ***2.5.2.4 Adoptive transfer***

A total of  $2 \times 10^6$  naive, Th1 KJ1.26<sup>+</sup> DO11.10 T cells were transferred by intravenous (i.v) injection into BALB/c recipients (n=42). One day following adoptive transfer all recipients were immunized s.c with 100 µg of OVA in CFA (Sigma-Aldrich). One day prior to, the day of, and on each of two days following, adoptive transfer, some of the recipients were injected s.c with 200 µg of 120G8 pDC depleting antibody (n=6) or 200 µg of the irrelevant isotype control YCATE.55.9.1 rat anti-dog CD8 IgG<sub>1κ</sub> (n=6). These mice did not receive further injections of 120G8 depleting antibody or isotype control for the remainder of the model.

#### ***2.5.2.5 Arthritis induction and monitor***

Ten days after immunisation with OVA in CFA, all animals were challenged by s.c. injection close to the left ankle joint with 100 µg of heat-attenuated OVA (HAO) in 50 µl of saline. One day prior to, the day of, and on each of two days following, challenge, some of the recipients were injected s.c with 200 µg of 120G8 pDC depleting antibody (n=10), 200 µg of the isotype control YCATE.55.9.1 rat anti-dog CD8 IgG<sub>1κ</sub> (n=10) or 200 µl PBS (n=10). pDC depletion was measured in one animal from each group by harvesting lymph nodes and spleen, and analyzing mPDCA-1 (Miltyeni Biotec), B220 and CD11c (both R&D) cell expression by flow cytometry. Mice were subsequently monitored for signs of arthritis. Footpad thickness was measured daily with a dial caliper (Kroeplin). At day 7 following challenge mice were sacrificed and for histological assessment hind limbs were removed and fixed in 10% neutral-buffered formalin and 6-µm sections were stained with haematoxylin and eosin (H&E) or toluidine blue (both from Sigma-Aldrich). Immunohistochemical analysis was carried out by Colin Hughes, University of Glasgow. For *ex vivo* antigen-specific assays and analysis of Foxp3<sup>+</sup> Tregs, draining popliteal lymph nodes were harvested from each mouse and processed immediately (2.4.3.3). To determine serum antibody levels, blood was also taken from each mouse and centrifuged at

800 g for 20 minutes. Serum was then taken and placed in a fresh tube at -20°C until analysis (2.5.2.7).

#### ***2.5.2.6 Measurement of antigen-specific response ex vivo***

Antigen-specific responses were assessed by proliferation and cytokine production. Proliferation of Ag-specific T cells was assessed by the tritiated thymidine uptake assay to evaluate DNA synthesis. Briefly, at the times indicated, lymph nodes were removed and single cell suspensions prepared and plated in triplicate at  $1 \times 10^5$  cells/well in round bottom 96 well plates in complete medium. Cells were stimulated, as indicated, with or without OVA (1 mg/ml) (Worthington) or bovine collagen type II (CII) (50 µg/ml) (Sigma). The final volume in wells was 200 µl. Cultures were incubated at 37°C 5% (v/v) CO<sub>2</sub> atmosphere at 95% humidity. At 72 hours 100 µl of supernatant was removed from each well and stored at -20°C for later cytokine analysis by multiplex (2.7.2). To the cultures was added 100 µl of complete medium (as above) supplemented with 0.5 µCi/well [6-3<sup>H</sup>]-thymidine (Amersham Pharmacia Biotech) for the last 8 hours of culture to allow incorporation into cellular DNA. Cells were harvested onto glass fibre filter mats (Perkin Elmer) using a Betaplate 96-well harvester (Amersham Pharmacia Biotech). Incorporated label was assessed by liquid scintillation counting and results are expressed as the Index of Stimulation, calculated as the proliferation in triplicate wells supplemented with OVA or CII divided by the proliferation in triplicate cultures in media alone.

#### ***2.5.2.7 Detection of serum antibodies from adoptive transfer recipients***

To detect B cell derived anti-OVA, anti-collagen and anti-IgG<sub>2a</sub> in serum, Nunc-Immuno plates (Nalge Nunc International) were coated with 100 µl of 20 µg/ml OVA, 2 µg/ml CII or 1 µg/ml IgG<sub>1</sub> (BD Pharmingen) in carbonate buffer (Sigma-Aldrich) overnight at 4°C. Plates were then washed in PBS/0.5% Tween20 three times prior to blocking PBS/10% FCS for 1 hour at 37°C. Plates were washed once again before incubation with serially diluted samples (in PBS) for 2 hour at 37°C. After further washing, plates were incubated in goat anti-mouse IgG<sub>2a</sub>-HRP (1/10000; Southern Biotech) for 1 hour at 37°C. Plates were washed once again and 100 µl tetramethylbenzidine (TMB) added to each well (Biosource). Colour reaction was stopped using 100 µl Stop solution (Biosource), and optical density (OD) analysed read on a plate reader at 570 nm.



## **2.6 Flow Cytometry Methods**

### **2.6.1 Enumeration of DC subsets in PB and SF**

Circulating DC populations were identified by flow cytometry using a FACScalibur (Becton Dickinson) and using a circulating DC enumeration kit (Miltenyi Biotech) according to manufacturer's instructions (Blood Dendritic Cell Enumeration Kit, Miltenyi Biotech). Briefly, a white blood cell (WBC) count was calculated from PB and SF samples using a Coulter Counter (Beckman Coulter). Aliquots of whole blood were labelled with a cocktail of antibodies including anti-CD14-phycoerythrin (PE).Cy5 and anti-CD19-PE.Cy5 plus or minus anti-CD1c-PE (mDC marker) and CD303 (BDCA-2)-fluorescein isothiocyanate (FITC) (pDC marker) or mouse IgG<sub>2a</sub>-PE and mouse IgG<sub>1</sub>-FITC antibodies. A proprietary dead cell discriminator was also included. After incubation, red cell lysis and wash, cells were fixed and examined using a FACScalibur and analysed using Cellquest software (both Becton Dickinson). Results were expressed as % mDC or pDC in WBC or as absolute numbers per ml of PB/SF calculated as (% positive - % negative cells) x WBC per ml ( $\times 10^6/100$ ).

### **2.6.2 Analysis of mDC and pDC phenotype and maturation**

Isolated DC were phenotyped by four colour flow cytometry using the following monoclonal antibodies against: CD1c-FITC (IgG<sub>2a</sub>) (mDC only), CD303 (BDCA-2)-FITC (IgG<sub>1</sub>) (pDC only) (both Miltenyi Biotec), CD123 (IL-3R $\alpha$ )-PE (IgG<sub>1</sub>) (pDC only) (Becton Dickinson), CD11c-PE (IgG<sub>1</sub>) (mDC only), CD14-PE (IgG<sub>2a</sub>) (mDC only), CD40-APC (IgG<sub>1</sub>), CD62L-FITC (IgG<sub>1</sub>), CD80-FITC (IgG<sub>1</sub>), CD83 (B7-1)-APC (IgG<sub>1</sub>), CD86 (B70/B7-2)-APC (IgG<sub>1</sub>), CCR7-PE (IgG<sub>2a</sub>) or the relevant isotype controls as indicated (all BD Biosciences Pharmingen). Isolated T cells were phenotyped by using CD3-FITC (IgG<sub>1</sub>) and CD4-PE (IgG<sub>1</sub>; both BD Biosciences Pharmingen). Isolated monocytes were phenotyped by using CD14-FITC (IgG<sub>2a</sub>; BD Biosciences Pharmingen). A proprietary dead cell discriminator (Viaprobe; BD Biosciences Pharmingen) was also included. Briefly, pDC or mDC were washed in PBS/0.1% NaN<sub>3</sub>/0.2% BSA, followed by incubation in the above monoclonal antibodies, as indicated, for 20 minutes on ice. Cells were then washed 3 times in buffer, resuspended and analysed for surface expression of the indicated phenotype and maturation markers by flow cytometry.

### **2.6.3 Analysis of IL-15 on BDB2**

#### **2.6.3.1 IL-15R $\alpha$ expression**

The expression of IL-15R $\alpha$  was first determined on BDB2 cells by incubation with the IL-15R $\alpha$  specific M161 (Genmab). Briefly, BDB2 cells were washed in PBS/0.1% NaN<sub>3</sub>/2% goat serum and resuspended cells incubated in 10  $\mu$ g/ml M161 or isotype control for 30 minutes on ice. After centrifugation at 300 g for 5 minutes, cells were resuspended in PBS/0.1% NaN<sub>3</sub>/2% goat serum and PE-conjugated goat anti-mouse IgG for a further 30 minutes on ice. After further centrifugation, the cells were resuspended and surface expression of IL-15R $\alpha$  determined by flow cytometry.

#### **2.6.3.2 Surface-bound IL-15 detection**

Surface-bound IL-15 was detected on BDB2 cells by flow cytometry. Briefly, BDB2 cells were incubate in 100 ng/ml IL-15 (R&D) for 30 minutes at 37°C. Cells were then immediately washed in ice-cold PBS. Cells were blocked by incubation in PBS/0.1% NaN<sub>3</sub>/2% goat serum for 30 minutes on ice. After washing at 4°C, cells were incubated in 7  $\mu$ g/ml F(ab')<sub>2</sub> anti-IL-15 or 7  $\mu$ g/ml F(ab')<sub>2</sub> human IgG isotype control for 30 minutes on ice. Cells were washed once again followed by incubation with FITC-conjugated goat anti-human IgG (Fab) (Sigma-Aldrich). After further centrifugation, the cells were resuspended and surface expression of IL-15 determined by flow cytometry.

### **2.6.4 pDC depletion from BALB/c**

Depletion of pDC from BALB/c after 120G8 s.c injection (2.5.2.4) was assessed by flow cytometry methods. Briefly, lymph nodes and spleen were harvested from animals and processed as described (2.4.2.3). Single cell suspensions were resuspended in PBS/0.1% NaN<sub>3</sub>/0.2% BSA and stained with mPDCA-1-FITC (Rat IgG<sub>1</sub>; Miltenyi Biotec) or CD11c-FITC (Ham IgG<sub>2a</sub>)/B220-PE (Rat IgG<sub>1</sub>) or appropriate isotype control (all BD Biosciences) for 20 minutes on ice. After washing in PBS three times, cells were resuspended in FACS buffer and fluorescence measured on a FACScalibur (Becton Dickinson).

### **2.6.5 Intracellular cytokine staining of Th1 differentiated Tg OVA<sub>323-339</sub> TcR DO11.10 CD4<sup>+</sup> T cells**

Intracellular cytokine analysis for IFN- $\gamma$  production in Tg OVA<sub>323-339</sub> TcR DO11.10 CD4<sup>+</sup> T cells was carried out prior to transfer to Balb/c recipients. Cells were harvested from

culture and centrifuged at 1500 rpm for 5 min and washed 3 times in FACS buffer. They were then stained with FITC labeled KJ.126 antibody for 20 min at 4°C and washed 3 times with FACS buffer. The cells were then resuspended in 100 µl Cytofix/Cytoperm (BD Pharmingen) for 20 min at 4°C and washed twice with Perm Wash (BD Pharmingen). Permeable cells were subsequently stained with PE labeled anti-IFN- $\gamma$  (BD Pharmingen) for 30 min at 4°C. Control cells were incubated with relevant isotype control antibodies. Cells were finally washed 3 times in FACS buffer, resuspended and analysed for intracellular IFN- $\gamma$  by flow cytometry, using a FACScalibur.

#### **2.6.6 Identification of Foxp3<sup>+</sup>T regs**

Intracellular analysis of Foxp3 in CD4<sup>+</sup> T cells was carried out after harvesting lymph nodes from BALB/c undergoing transient arthritis. Single cell suspension were prepared from lymph nodes and centrifuged at 1500 rpm for 5 min and washed 3 times in FACS buffer. They were then stained with FITC-conjugated CD4 antibody for 20 min at 4°C and washed 3 times with FACS buffer. The cells were then resuspended in 100 µl Cytofix/Cytoperm (BD Pharmingen) for 20 min at 4°C and washed twice with Perm Wash (BD Pharmingen). Permeable cells were subsequently stained with PE labeled anti-FoxP3 (eBioscience) for 30 min at 4°C. Cells were finally washed 3 times in FACS buffer, resuspended and analysed for intracellular Foxp3 expression by flow cytometry.

### **2.7 Cytokine analysis**

#### **2.7.1 Enzyme-Linked-ImmunoSorbent Assay (ELISA)**

Cytokine levels in culture supernatants were analyzed by sandwich ELISA. Sandwich ELISA involves attachment of a capture antibody to a 96 well plate followed by culture samples in a buffer, which minimizes sample attachment to the 96 well plate. An enzyme-labeled antibody is then added for detection. Levels of IFN- $\alpha$  in pDC culture supernatants and SF samples were measured using a human IFN- $\alpha$  Matched Antibodies Module Set (Bender MedSystems®) according to the manufacturer's instructions. Levels of IFN- $\gamma$  and TNF- $\alpha$  in PBMC, SFMC, mDC and pDC culture supernatants were assessed using a human IFN- $\gamma$  Cytoset<sup>TM</sup> and Human TNF- $\alpha$  Cytoset<sup>TM</sup>, respectively. Briefly, 96 well NUNC MaxiSorp microplates were coated in 100 µl per well PBS/coating antibody (IFN- $\alpha$  10 µg/ml; IFN- $\gamma$  4 µg/ml; TNF- $\alpha$  2 µg/ml) for 12-18 hours at 4°C. Wells were then

aspirated and blocked in 300 µl per well PBS/0.5%BSA for one hour at room temperature on an orbital shaker. Following vigorous washing in PBS/0.5%polyoxyethylene sorbitan monolaurate (Tween20) (Sigma-Aldrich), to each well was then added 100 µl of either the sample of interest, or standards as supplied, followed by 50 µl of biotinylated detection antibody (IFN- $\gamma$ , 0.4 µg/ml; TNF- $\alpha$ , 0.8 µg/ml) or horse-radish peroxidase (HRP)-conjugate for IFN- $\alpha$  detection only, and incubated for 2 hours at room temperature with shaking. After 4 washes in PBS/0.5%Tween20, 100 µl/well of Streptavidin-HRP at 1/5000 in PBS was added (not IFN- $\alpha$  detection plates) and incubated for 30 minutes at room temperature. After washing, 100 µl of chromogen tetramethylbenzidine (TMB) (Biosource) was added to all wells. Colour change was monitored and stopped by addition of 100 µl/well stop solution (Biosource). The optical density of plates was measured at 450 nm (reference filter 650 nm).

### **2.7.2 Multiplex Bead Assay**

Cytokine production was analysed by using Multiplex Bead Assay (Biosource, UK) according to the manufacturer's instructions. In human cell cultures a 20-plex was used containing the chemokines and cytokines CCL2 (MCP-1), CCL3 (MIP-1 $\alpha$ ), CCL4 (MIP-1 $\beta$ ), CCL5 (RANTES), CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), IL-7, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , GM-CSF, IFN- $\gamma$ , IL-10, IL-17, IL-1R $\alpha$ , IL-2, IL-2R, IL-4, IL-5, IL-12p40, IL-13, IL-15, IFN- $\gamma$ . In murine cell cultures a 10-plex was used containing the cytokines GM-CSF, IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, TNF- $\alpha$ . The principle of this method is a sandwich immunoassay where specific antibodies have been coated to the surface of fluorescently encoded microspheres. Each microsphere is labeled with a distinguishable fluorophore that allows it to be assigned or gated to a particular region by the scanner. Cytokine capture microspheres are first incubated with the culture supernatant under investigation followed by biotinylated detection antibody. Relevant standards are also used to enable quantitative analysis. Finally, Streptavidin-RPE is added and the fluorescence bound to the microspheres analysed using a Luminex XMAP<sup>TM</sup> system. The fluorescence intensity is proportional to the concentration of cytokine/chemokines present in the sample.

## **2.8 Immunohistochemical Methods**

### **2.8.1 Antibodies**

The following primary mouse monoclonal antibodies (mAbs) were used: anti-CD1c (IgG<sub>2a</sub>; BD Beckman Coulter), biotin-conjugated anti-CD1c (BDCA1; IgG<sub>2a</sub>, Miltenyi Biotec, Bergisch, Gladbach, Germany), biotin-conjugated anti-CD304 (BDCA4; IgG<sub>1</sub>, Miltenyi Biotec), anti-CD1c (BDCA1; IgG<sub>2a</sub>, Miltenyi Biotec), anti-CD304 (BDCA4; IgG<sub>1</sub>, Miltenyi Biotec), FITC-conjugated anti-CD1c (BDCA1; IgG<sub>2a</sub>, Miltenyi Biotec), FITC-conjugated anti-BDCA2 (IgG<sub>1</sub>, Miltenyi Biotec), anti-CD11c (IgG<sub>1</sub>, BD Pharmingen, San Diego, CA), anti-CD123 (IgG<sub>1</sub>; BD Biosciences Pharmingen), PE-conjugated anti-CD123 (IgG<sub>1</sub>, BD Biosciences), anti-CD83 (IgG<sub>1</sub>, BD Pharmingen), anti-CD3 (IgG<sub>1</sub>, BD Biosciences), anti-CD8 (IgG<sub>1</sub>, BD Biosciences), anti-IL-12p70 (IgG<sub>1</sub>, R&D Systems, Minneapolis, MN), anti-IL-15 (IgG<sub>1</sub>, Diaclone SAS), anti-IL-18 (IgG<sub>1</sub>, MD Biosciences, St. Paul, MN), and anti-IFN- $\alpha$  and IFN- $\beta$  (both IgG<sub>1</sub>, PBL Biomedical Laboratories, Florence, Italy). Polyclonal rabbit anti-human IL-23p19 subunit was kindly provided by Dr. J. Pirhonen (Department of Microbiology, National Public Health Institute, Helsinki, Finland).

### **2.8.2 Single Immunohistochemical Staining**

Acetone-fixed cryosections were incubated with mAbs against CD1c (BDCA1) or CD304 (BDCA4) for 1 h at room temperature after blocking endogenous peroxidase activity with H<sub>2</sub>O<sub>2</sub> and sodium azide (NaN<sub>3</sub>). As negative controls, the primary antibodies were omitted or irrelevant isotype-matched antibodies were applied. After incubation with goat anti-mouse horseradish peroxidase (HRP)-conjugated (DakoCytomation) for 30 min at room temperature, sections were incubated for 15 min with biotinylated tyramine (Perkin Elmer), followed by an incubation with streptavidin-HRP (strep-HRP; DakoCytomation). Peroxidase activity was revealed using amino-ethylcarbazole (AEC) substrate kit (SK-4200, Vector laboratories). With this procedure CD1c (BDCA1)<sup>+</sup> mDC and CD304 (BDCA4)<sup>+</sup> pDC stained red. Other cellular elements were counterstained with hematoxylin (Sigma-Aldrich), dried and mounted with glycerol/gelatine.

### **2.8.3 Double Immunohistochemical Staining**

The double staining of CD1c or CD304 with CD3, CD8, CD83 and DC-LAMP was undertaken by Dr M.C.Lebre, AMC, Amsterdam, The Netherlands.

For double immunohistochemical stainings with CD1c (BDCA1) or CD304 (BDCA4) in combination with CD3 or CD8 respectively, the sections were incubated overnight at 4°C with either biotin-conjugated anti-CD1c or with biotin-conjugated anti-CD304 as primary mAbs. After incubation with strep-HRP for 30 min at room temperature sections were incubated for 15 min with biotinylated tyramine, followed by incubation with strep-HRP for 30 min. Following a 15 min incubation with 10% normal mouse serum, anti-CD3 or anti-CD8 were applied to the sections and incubated for 1 h at room temperature followed an incubation with alkaline phosphatase (AP)-conjugated goat anti-mouse for 30 min. Peroxidase activity was revealed as stated above and AP activity was revealed using alkaline phosphatase substrate III kit (Vector laboratories).

For double immunohistochemistry of CD304 in combination with CD123, acetone-fixed cryosections were incubated in 0.5% H<sub>2</sub>O<sub>2</sub>/methanol for 30 minutes followed by blocking in 20%horse serum/20%human serum/PBS (Sigma-Aldrich) for 30 minutes at room temperature. Sections were then incubated with mAb against CD123 (IL-3R $\alpha$ ) for 90 minutes at room temperature. As negative controls mouse IgG<sub>1</sub> was applied. After washing, slides were incubated with biotinylated horse anti-mouse IgG (H+L) (Dakocytomation). Slides were then incubated with Vectastain ABC (Vector) for 30 minutes followed by 5 minute incubation with DAB Ni (Vector). CD123<sup>+</sup> cells stain grey. Sections were then incubated for 90 minutes at room temperature with CD304. Immunohistochemistry was then carried out as above, but NovaRED<sup>TM</sup> (Vector) was used for visualization. Other cellular elements were counterstained with Harris modified haematoxylin solution (Sigma-Aldrich), followed by alcohol dehydration and mounting in DPX mountant for microscopy (BDH Laboratory Supplies). By this procedure CD304<sup>+</sup> cells stain red, whereas CD304<sup>+</sup>/CD123<sup>+</sup> cells are black.

### **2.8.4 Immunofluorescent Staining**

For double immunofluorescence staining, sections were first incubated with FITC-conjugated primary mAbs against CD1c or CD303 (BDCA2) followed by incubation with

rabbit anti-FITC (DakoCytomation) and with Alexa-488 conjugated goat anti-rabbit (Molecular Probes Europe). After blocking with normal mouse serum, the sections were incubated with the mouse monoclonal antibodies against CD83, IL-12p70, IL-15, IL-18, IFN- $\alpha$  or IFN- $\beta$ , or with the rabbit polyclonal against IL-23p19. After incubation with Alexa-594-conjugated goat anti-mouse or with Alexa-594-conjugated goat anti-rabbit (Molecular Probes Europe), the slides were analyzed using a fluorescence microscope (Leica DMRA) coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components). For coexpression of CD83 or DC-LAMP and mDC or pDC 12 patients with RA, 5 patients with OA and 7 patients with PsA were analyzed. For the expression of cytokines by mDC and pDC 5 patients with RA were analyzed. To quantify the data, the numbers of double positive staining cells were counted in a minimum of 6 microscopic fields and the percentage of double positive cells was calculated. The selection of these patients was based on the presence of mDC and/or pDC in the synovium.

#### **2.8.5 Digital Image Analysis**

All sections were coded and randomly analyzed by computer-assisted image analysis. For all markers, 18 high-power fields were analyzed. The high-power field images were acquired on an Olympus microscope (Olympus), captured using a charged coupled device video camera (Sony), and digitized using a PV100 multimedia 16-bit colour video digitizer card, using a standardized macro program to simplify as well as to standardize the acquisition process. The resultant colour images were in a 640x480 pixel RGB format with a 24-bit resolution, enabling the use of 16581375 colours. For each acquisition session, the microscope, camera and computer were calibrated according to a standardized procedure. The images obtained were stored as bitmaps without compression using a Zip disk and portable driver (Iomega, Roy, UT, USA). The images of the high-power fields were analyzed using the Qwin Pro V2.2 analysis system (Leica Microsystems), which consists of a personal computer (PC) with software (Intel Pentium 200 MHz processor and Windows<sup>TM</sup> NT 4.0 environment).

#### **2.9 Statistical analysis**

Data are expressed as the median and interquartile range or mean  $\pm$  standard error of the mean (SEM) as indicated. Where presented statistical significance was determined using

the nonparametric Mann-Whitney U test between disease groups and healthy controls. CRP correlation to PB DC numbers was analysed by Pearson Correlation. A  $p$  value  $<0.05$  was considered significant.



## **2.10 Suppliers addresses**

### **Amersham Pharmacia Biotech**

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### **BD Biosciences**

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### **Bender MedSystems®**

#### **C/o Catlag-MedSystems**

Botyl Rd  
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Buckingham MK18 2LR

### **Cadisch Precision Meshes Ltd**

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879 High Rd  
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London N12 8QA

### **Calbiochem**

#### **c/o CN Biosciences**

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Beeston  
Nottingham NG9 2JR

### **Chondrex Inc**

#### **C/o MD Biosciences**

Morwell Diagnostics GmbH  
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Postfach 8132  
Zürich Switzerland

### **Corning Incorporated**

Corning NY 14881  
USA

### **Dako UK Ltd**

Denmark House  
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Cambridgeshire CB7 4ET

### **Diaclone SAS**

#### **C/o I.D.S**

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Tyne and Wear NE35 9PD

### **eBiosciences**

#### **C/o Insight Biotechnology Ltd**

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### **Genosys**

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**Gibco**

Paisley PA4 9RF  
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**Harlan UK Ltd**

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**Invitrogen Life Technologies**

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Paisley

**InvivoGen**

**C/o Autogen Bioclear UK Ltd**  
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Calne, Wiltshire SN11 DPY

**Kroeplin**

**C/o Newman Tools Inc**  
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**Leica Microsystems (UK) Ltd**

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**Leo Laboratories Ltd**

Prnces Risborough  
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**Medicell International Ltd**

239 Liverpool Rd  
London N1 1LX

**Miltenyi Biotec**

Almac House  
Church Lane  
Bisley  
Surrey GU24 9DR

**Millipore**

Billencia  
MA 01821  
USA

**PeproTech EC Ltd**

29 Margravine Rd  
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**Perkin Elmer**

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**StemCell Technologies SARL**

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### **Chapter 3: Enumeration, phenotype and functional analysis of distinct dendritic cell subsets in psoriatic arthritis and rheumatoid arthritis**

### 3.1 Introduction

The inflammatory arthritides, including rheumatoid arthritis (RA) and psoriatic arthritis (PsA), comprise autoimmune disorders characterised by chronic joint inflammation, immune cell infiltration to the synovium, fibroblast-like synoviocyte expansion and destruction of cartilage and bone. Extensive *in vivo* and *in vitro* studies have identified multiple pro-inflammatory cytokines and enzymes implicated in the pathogenesis of both of these disorders. Targeting cytokines has proven therapeutically useful exemplified particularly in tumour necrosis factor (TNF) blockade and more recently in targeting interleukin (IL)-6, and IL-15 (339, 340, 342, 578). Cytokine blockade however exhibits variable responses across patient populations and importantly disease activity recurs upon cessation of therapy. A major challenge in therapeutics now is to develop strategies that re-establish immune tolerance such that amelioration of inflammation is accompanied by long-term disease suppression. Early evidence that such responses may be feasible is derived from clinical studies utilising CTLA-4Ig (381) and anti-CD20 (289, 579) in RA and co-stimulatory blockade in psoriasis (571, 580, 581).

Many data indicate the presence and significance of autoimmune processes in articular inflammation. In RA, recent results implicate citrullinated peptides in disease progression and response to TNF blockade (311). Additional auto-specific antigens include type II collagen (300) and cartilage derived gp39 (298, 299). Although in PsA few autoimmune targets have been identified, perhaps reflecting the absence thus far of defined autoantibodies, analysis of PsA synovial and skin T cell subsets clearly indicate oligoclonal responses that may be self targeted (582, 583). Critically, in both diseases, upstream immunological processes have been poorly characterised *ex vivo*, particularly with respect to the site and nature of antigen presentation. Antigen presenting macrophages and B cells play a potential role in synovial inflammation, especially in mediating cartilage degradation (584, 585). However, the role played by various subsets of dendritic cells (DC) is less clear (295). Although enrichment of DC in RA synovial tissue and fluid and in seronegative synovial fluid has been reported (274, 530-535), the considerable phenotypic, morphologic and functional variations between DC subsets related to lineage, stage of maturation and tissue localisation remains less well understood.

This partly reflects difficulty in adequately identifying DC subsets in peripheral blood and tissues where most methodologies rely on complex combinations of surface markers.

Recently, novel markers useful in human DC studies have been defined that resolve these issues (397). Five distinct subsets of lineage negative, HLA-DR<sup>+</sup> DC have been classified in humans. Four subsets, CD1b/c<sup>+</sup>, CD16<sup>+</sup>, CD141 (blood dendritic cell antigen (BDCA)-3)<sup>+</sup> and CD34<sup>+</sup> are myeloid-lineage derived, while the fifth, CD123 (IL-3Rα<sup>+</sup>)/ CD303 (BDCA-2)<sup>+</sup>, has a lymphoid phenotype and is described as plasmacytoid DC (pDC) (397, 398). Comprising 0.5-1.0% of all circulating mononuclear cells, myeloid DC (mDC) are characterised as CD1c<sup>+</sup>/ CD11c<sup>+</sup>/ CD45RO<sup>+</sup>/ HLA-DR<sup>+</sup>/ CD123 (IL-3Rα)<sup>lo</sup>. Capable of IL-12-p70, IL-6, TNF-α and IL-10 production in response to bacterial or CD40L stimulation as well as antigen capture and presentation (409), mDC require GM-CSF for survival *in vitro* and can differentiate into interstitial DC (int DC) and Langerhans cells (LC) in the presence of GM-CSF, IL-4 and TGF-β (410). Conversely pDC, characterised as CD303 (BDCA-2)<sup>+</sup>/ CD304 (BDCA-4)<sup>+</sup>/ CD123 (IL-3Rα)<sup>high</sup>/ CD11c<sup>-</sup>/ HLA-DR<sup>+</sup>/ CD45RA<sup>+</sup>, comprise less than 0.3% of all circulating mononuclear cells and secrete large amounts of type I interferons in response to virus mediated stimulation (411, 412).

It has been suggested that DC play a role in the initiation and perpetuation of inflammatory arthritis by presentation of arthritogenic antigens to autoreactive T cells (273, 294-296). This presentation may drive aberrant memory T cell responses, promoting B cell activation and immunoglobulin class switching. DC infiltration into the synovium occurs early in disease pathology (295, 550, 552). DC could represent important cellular targets in inflammatory synovitis, by virtue of their potent antigen presentation potential and capacity to promote local inflammation via TLR expression (491, 586) and cytokine release (402, 412, 465, 498, 587).

### 3.2 Aims and Objectives

Recent advances in identifying DC subsets have improved resolution of subset heterogeneity. It was therefore the aim of this chapter to undertake a comprehensive enumeration and characterisation of the phenotype and function of pDC and mDC in peripheral circulation as well as synovial effusions in the context of inflammatory arthritis.

Specifically, I planned to address:

- Whether total peripheral circulating numbers of pDC and mDC are altered in inflammatory arthritis in comparison to OA patients and healthy donors, by use of a robust and sensitive assay system utilising specific receptors.
- Whether peripheral circulating pDC and mDC express a normal or abnormal phenotype compared to healthy donor derived pDC and mDC.
- Whether pDC and mDC are detectable in synovial effusion from RA and PsA and if so, the total number by volume of each.
- Whether pDC and mDC in synovial fluid from RA and PsA patients display an immature or mature phenotype.
- Whether an assay system could be established in order to determine if pDC and mDC in synovial fluid are hyper or hypo-responsive to TLR stimulation in comparison to healthy donor derived pDC and mDC, as assessed by cytokine release and maturation marker expression.
- Finally, whether there exist any identifiable and specific differences in the number, phenotype and function of pDC and mDC from RA blood and synovial fluid in comparison to PsA blood and synovial fluid, thus indicating a disease specific phenomenon.

### **3.3 Results**

#### **3.3.1 Patients**

Inflammatory arthritis patients attending Glasgow Royal Infirmary (GRI) fulfilled the American College of Rheumatology criteria for RA (23), or met diagnostic criteria for PsA as previously described (572, 573). All patients and healthy donors gave informed consent and the study protocol was approved by the Ethical Committee, GRI. Peripheral blood (PB) was obtained from patients with RA (n=12), PsA (n=13) and OA (n=11) and compared to healthy controls (n=12). Matched synovial fluid (SF) was obtained from a subset of these donors (RA=6, PsA=6) (Table 3.1). All cell analysis was undertaken on freshly isolated cells. For cytokine investigation, cell free SF and supernatants from cell culture were stored in the Centre for Rheumatic Disease (CRD) Biobank at  $-70^{\circ}\text{C}$  until analysed. At the time of study, RA patients were receiving methotrexate (n=7), sulphasalazine (n=8) or hydroxychloroquine (n=4) or a combination of these agents (n=5). PsA patients were receiving either methotrexate (n=11) or sulphasalazine (n=4) or a combination of these agents (n=2). OA patients did not receive any immune modulatory therapy, but were taking atenolol (n=3), omeprazole (n=3), simvastatin (n=2), arthrotec (n=1), zopiclone (n=1) or a combination of these agents (n=3). Patients on oral steroids were excluded from the study.

#### **3.3.2 Whole blood enumeration of circulating peripheral blood pDC and mDC**

We first chose to examine systemic circulating and local levels of DC subsets in inflammatory arthritis. Changes in circulating DC subpopulations have been identified in other autoimmune disease, particularly systemic lupus erythematosus (SLE) (498, 588-590). Until recently however, the quantitative analysis of DC subsets has been hindered by complex methodologies, a lack of specific surface markers and low absolute DC levels eluding reliable detection. We therefore utilised the novel DC markers CD1c (mDC) and CD303 (pDC), within a unique whole blood three colour FACS assay system allowing enumeration of DC numbers with high sensitivity. Fresh PB was obtained, a white blood cell (WBC) count performed, and the PB processed (as described in 2.6.1). The first step of analysis was to exclude dead cells and cellular debris by gating on the leukocyte fraction as delineated by forward scatter (FSC) and side scatter (SSC) (Fig. 3.1a). The CD1c expressing monocytes and B cells were excluded based upon CD14 expression (monocytes) or CD19 expression (B cells). Any remaining dead cells were excluded using



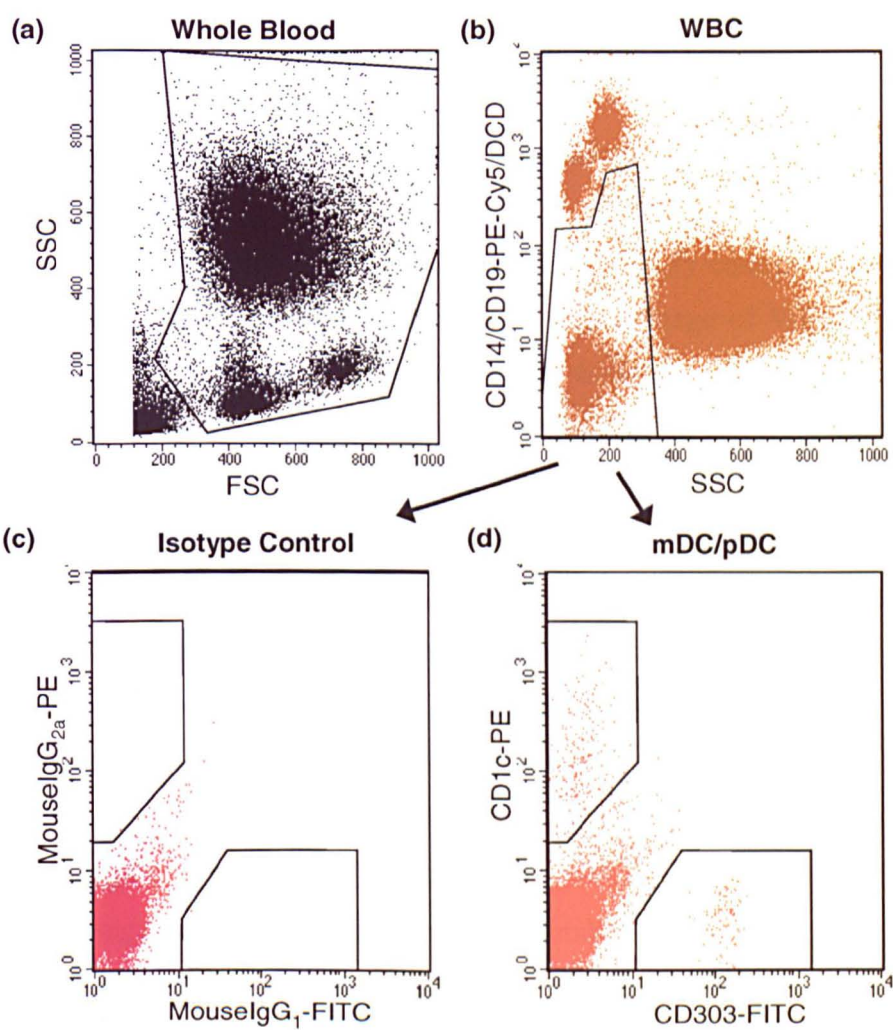
**Table 3.1. Patient data**

	Characteristic	PB study group	SF study group
RA	Age, Mean $\pm$ SD	58 $\pm$ 12 (24-80)	59 $\pm$ 15 (33-70)
	Female:Male	10:2	5:1
	WBC, Mean $\pm$ SEM	6.4x10 <sup>6</sup> /ml $\pm$ 1.2x10 <sup>4</sup> /ml	
	CRP (mg/liter)	26 $\pm$ 6 (6-100)	22 $\pm$ 7 (6-41)
PsA	Age, Mean $\pm$ SD	42 $\pm$ 14 (24-81)	41 $\pm$ 15 (25-50)
	Female:Male	10:3	5:1
	WBC, Mean $\pm$ SEM	8.0x10 <sup>6</sup> /ml* $\pm$ 4.5x10 <sup>5</sup> /ml	
	CRP (mg/liter)	14 $\pm$ 4 (6-73)	30 $\pm$ 15 (6-73)
OA	Age, Mean $\pm$ SD	63 $\pm$ 12 (52-82)	
	Female:Male	4:6	
	WBC, Mean $\pm$ SEM	6.0 x10 <sup>6</sup> /ml $\pm$ 8.9x10 <sup>5</sup> /ml	
	CRP (mg/liter)	10 $\pm$ 3 (6-14)	
Healthy	Age, Mean $\pm$ SD	44 $\pm$ 11	
	Female:Male	6:6	
	WBC, Mean $\pm$ SEM	6.4x10 <sup>6</sup> /ml $\pm$ 4.7x10 <sup>5</sup> /ml	

Mean  $\pm$  standard error of the mean (range) is shown, CRP (C/Reactive Protein), \*p<0.05 compared to healthy control PB

**Figure 3.1 Assay to enumerate mDC and pDC from whole blood.**

Whole blood was stained with an antibody cocktail containing CD14/CD19-PE-Cy5 and dead cell discriminator (DCD) with either CD1c-PE/CD303-FITC or mouse IgG<sub>2a</sub>-PE/mouse IgG<sub>1</sub>-FITC and analysed by FACS following lysis of red blood cells. White blood cells (WBC) were selected (a), monocytes, B cells, neutrophils and dead cells eliminated (b) and CD1c<sup>+</sup> mDC and CD303<sup>+</sup> pDC identified (d) as compared to the isotype control (c). Analysis shown is representative of a typical experiment, in this case using a healthy control.



a dead cell discriminating (DCD) nuclear stain, and neutrophils were excluded based upon SSC profile. The gate to encompass remaining cells was set routinely high towards the CD14<sup>+</sup> monocytes as PB mDC sometimes express CD14 at low levels. PB mDC and pDC were identified as discrete CD1c<sup>+</sup> (mDC) or CD303<sup>+</sup> (pDC) expressing populations (Fig. 3.1d) as compared to the isotype controls (Fig. 3.1c). By using this method we were able to calculate the number of mDC and pDC as a percentage of WBC and the total number of mDC and pDC/ml of PB. This was considered a vital step in order to account for variations in other cell populations, hence unmasking any 'false' differences.

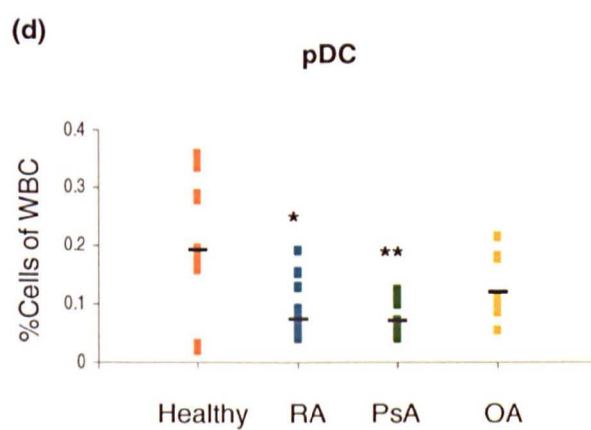
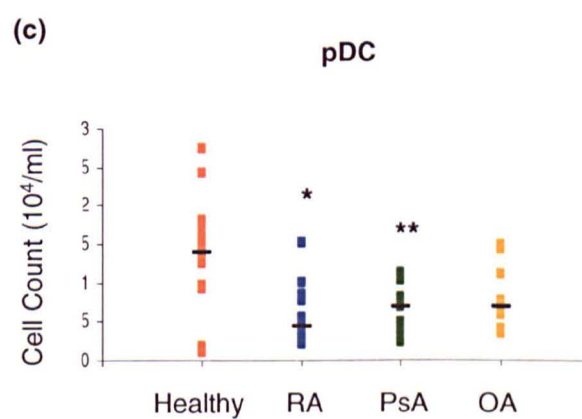
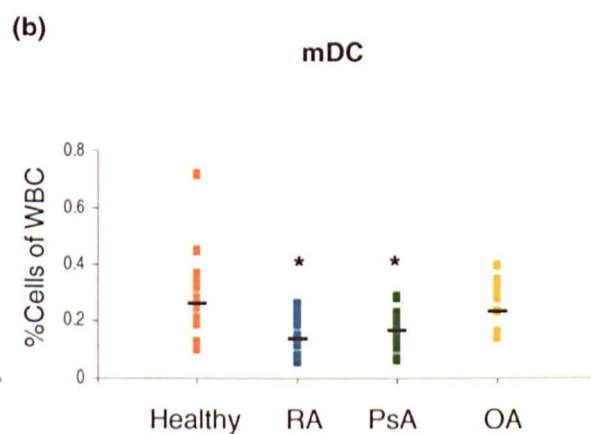
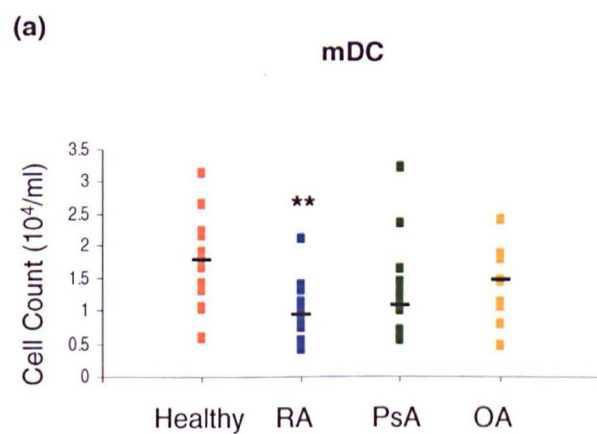
### **3.3.3 Number and proportion of circulating pDC are reduced in PsA and RA PB and mDC are reduced in RA PB.**

In RA PB, mDC were significantly decreased compared to healthy controls, as expressed as total numbers/ml PB (Fig. 3.2a) ( $p=0.0086$ ) and as a percentage of WBC (Fig. 3.2b) ( $p=0.0262$ ). In PsA PB, mDC were not significantly decreased compared to healthy controls when expressed as total numbers/ml in PB ( $p=0.1147$ ), but were significantly decreased when adjusted to percentage of WBC ( $p=0.018$ ) most likely reflecting an elevation in another cell population as indicated by a significantly increased WBC count in PsA PB compared to healthy controls (Table 3.1,  $p=0.0470$ ). In contrast, circulating pDC were significantly decreased in both RA and PsA PB compared to healthy controls expressed either as total numbers/ml PB (Fig. 3.2c) (RA,  $p=0.0194$ , PsA,  $p=0.0098$ ) or indeed as a percentage of WBC (Fig. 3.2d) (RA,  $p=0.0180$ , PsA,  $p=0.0051$ ). In OA PB, although there was a trend towards a decrease in pDC numbers/ml of PB, neither mDC nor pDC numbers were significantly reduced compared to healthy controls whether data were expressed as a percentage of total WBC or as total cell numbers/ml PB.

To determine if the observed reduction in circulating PB DC subsets was a cell specific phenomenon, or a pattern common to other myeloid or lymphoid lineage cells we also analysed the proportion and percentage of WBC of circulating CD14<sup>+</sup> monocytes (myeloid) and CD19<sup>+</sup> B cells (lymphoid). In RA and OA CD14<sup>+</sup> monocytes were not significantly decreased compared to healthy controls when expressed as total numbers/ml PB (Fig. 3.3a,  $p=0.3348$  and  $p=0.0756$  respectively) or when adjusted to percentage WBC (Fig. 3.3b,  $p=0.2318$  and  $p=0.0828$  respectively). Similarly, in PsA CD14<sup>+</sup> monocytes were not significantly decreased compared to healthy control when expressed as numbers/ml PB (Fig. 3.3a,  $p=0.3348$ ) but were significantly decreased when adjusted to

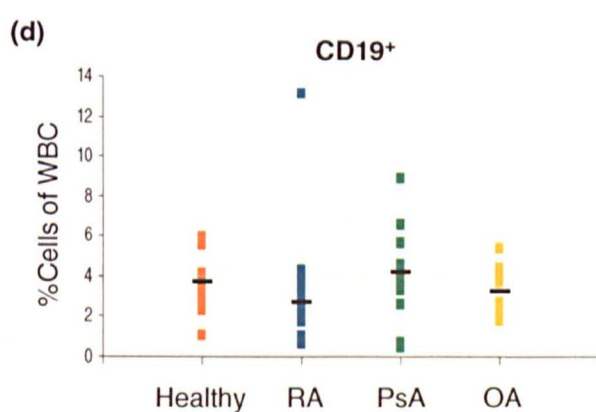
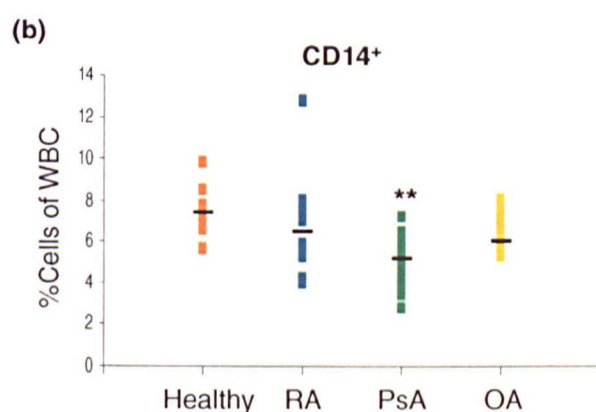
**Figure 3.2 Circulating pDC and mDC are significantly decreased in RA PB and pDC are significantly decreased in PsA PB.**

Total number of mDC (a) and pDC (c) per ml of PB and percentage of mDC (b) and pDC (d) of WBC in healthy (n=12), RA (n=12), PsA (n=13) and OA (n=11) subjects. Squares indicate individual samples, - = median, \*p<0.05, \*\*p<0.01. Statistical analysis was carried out using Mann Whitney.



**Figure 3.3 Circulating monocytes and B cells are not significantly decreased in RA, or OA PB but monocytes are decreased in PsA.**

Total number of CD14<sup>+</sup> monocytes (a) and CD19<sup>+</sup> B cells (b) per ml of PB and percentage of CD14<sup>+</sup> monocytes (c) and CD19<sup>+</sup> B cells (d) of WBC in healthy (n=12), RA (n=12), PsA (n=13) and OA (n=11) subjects. Squares indicate individual samples, - = median, \*\*p<0.01. Statistical analysis was carried out using Mann Whitney.





percentage WBC (Fig. 3.3b,  $p=0.0020$ ). Consistent with our previous observations, this significant decrease was once again most probably reflecting an elevation in another cell population. In RA, PsA and OA  $CD19^+$  B cells were not significantly decreased compared to healthy control when expressed as total numbers/ml in PB (Fig. 3.3c,  $p=0.214$ ,  $p=0.1571$  and  $p=0.2707$  respectively) or when adjusted to percentage of WBC (Fig. 3.3 d,  $p=0.0885$ ,  $p=0.5444$  and  $p=0.4887$  respectively).

### **3.3.4 Reduced PB DC subset numbers are inversely correlated to CRP**

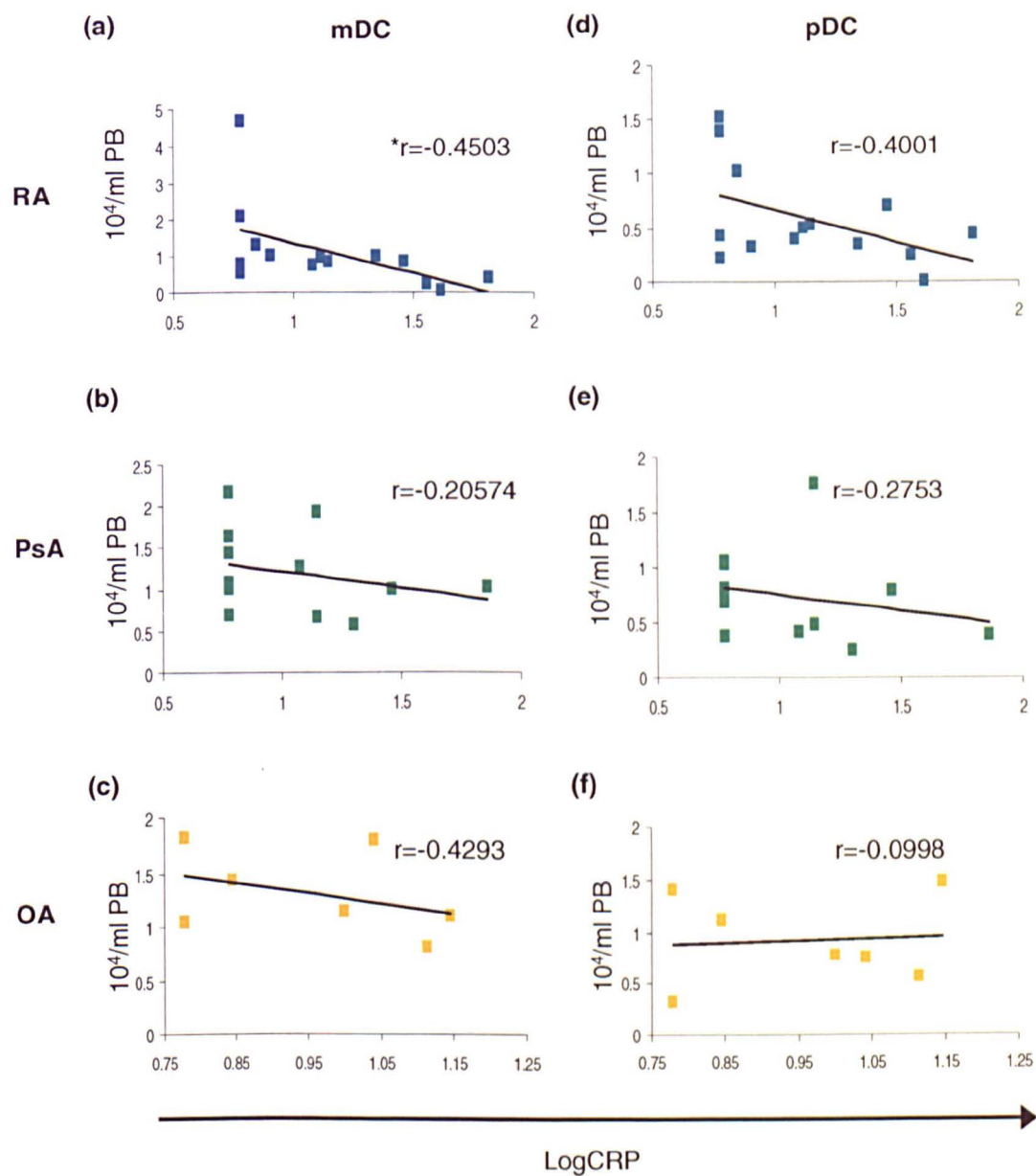
The significant decrease of pDC and mDC in RA PB and pDC in PsA PB, as well as the trend towards a decrease in pDC in OA PB was intriguing and led us to investigate a possible correlation to the magnitude of inflammatory disease activity. Accordingly, we correlated pDC and mDC numbers from PB of RA, PsA and OA patients with serum CRP concentrations. Pooled analysis of the disease subsets combined indicated a significant inverse correlation between mDC and pDC numbers/ml PB and serum CRP (mDC  $r=-0.346$ ,  $p=0.045$  and pDC  $r=-0.407$ ,  $p=0.017$ ), indicating a link between circulating DC subsets and inflammation. Disease subset analysis revealed that mDC numbers/ml PB ( $p=0.021$  and  $r=-0.45233$ ), but not pDC numbers/ml ( $p=0.228$  and  $r=-0.04001$ ), were significantly and inversely correlated to CRP levels from RA patients (Fig. 3.4a and 3.4d). However, neither mDC nor pDC numbers/ml PB were significantly inversely correlated to CRP levels from PsA patients ( $r=-0.20574$ ,  $p=0.496$  and  $r=-0.2753$ ,  $p=0.434$ ) (Fig. 3.4b and 3.4e) or OA patients ( $r=-0.4293$ ,  $p=0.238$  and  $r=-0.0998$ ,  $p=0.806$ ) (Fig 3.4c and 3.4f), indicating that RA dominates the analysis, most likely as CRP levels are lower in PsA patients.

### **3.3.5 CD62L is aberrantly expressed on RA and PsA circulating PB DC.**

To further study the phenotype of PB mDC and pDC, these cells were isolated by sequential positive selection in a subset of RA ( $n=5$ ) and PsA ( $n=5$ ) PB and compared to healthy controls ( $n=5$ ). The purity of the isolated DC was confirmed by double staining with CD1c and CD11c (mDC) (Fig. 3.5a) and CD303 and CD123 (pDC) (Fig. 3.5b) and was routinely  $>99\%$  mDC and  $>95\%$  pDC. Thereafter a panel of maturation markers was examined using FACS analysis. Both  $CD1c^+/CD11c^+$  mDC and  $CD303^+/CD123^+$  pDC from PsA and RA PB displayed an immature phenotype similar to mDC and pDC isolated from healthy controls (Fig. 3.5a and 3.5b respectively) with absent expression of the

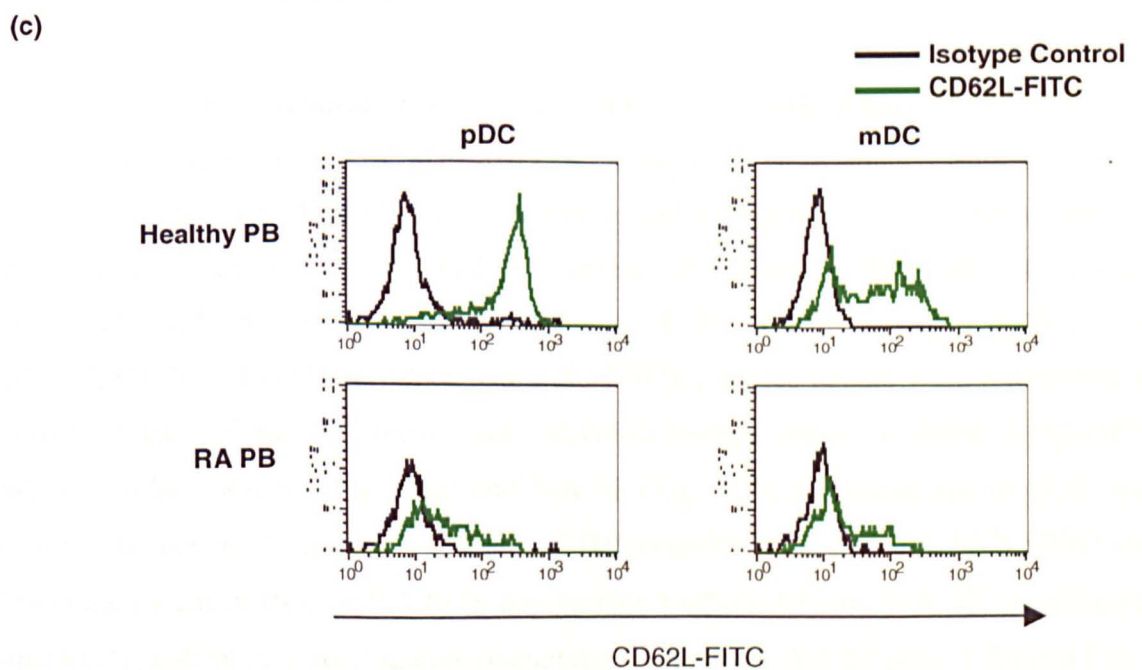
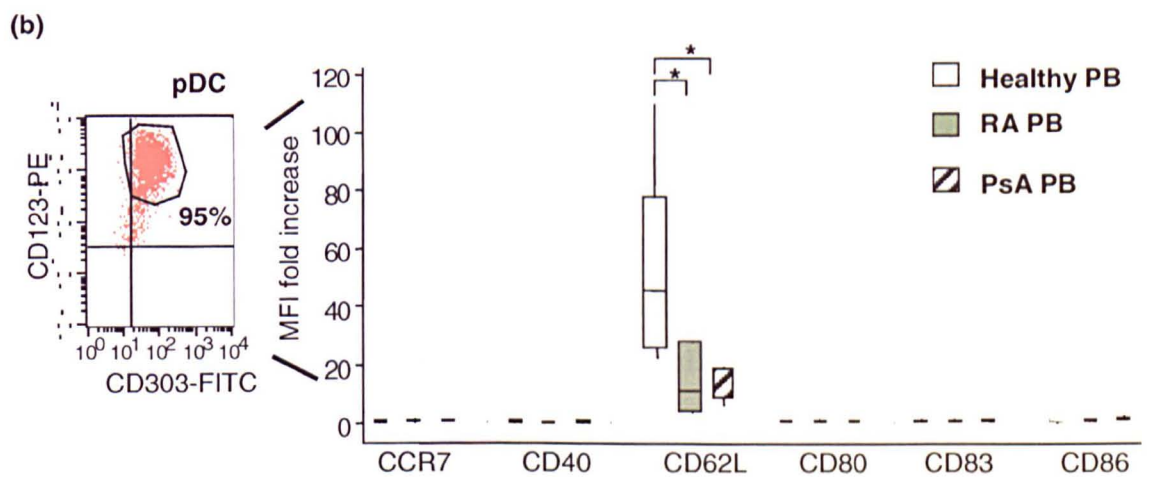
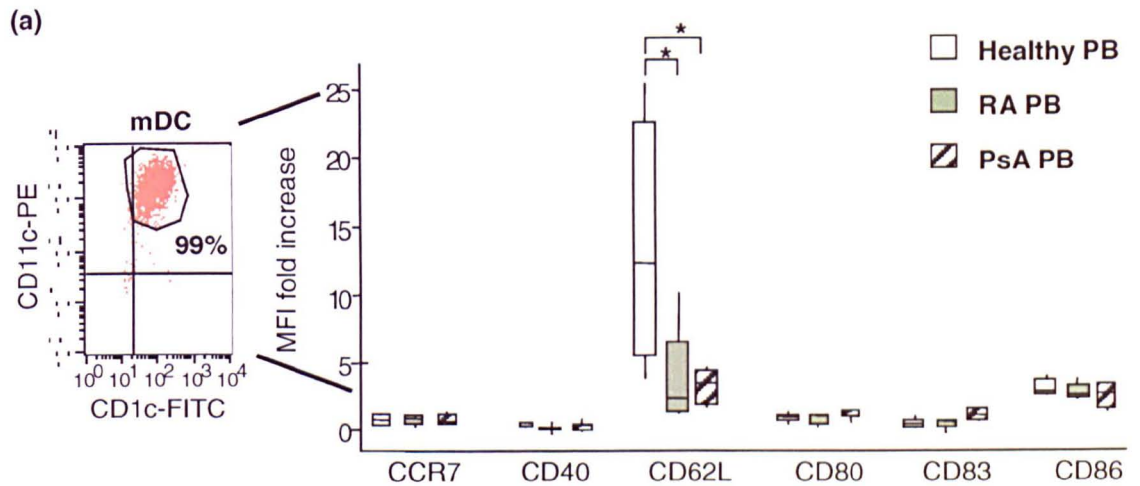
**Figure 3.4 Circulating mDC numbers are significantly inversely correlated to CRP levels in RA PB.**

The CRP levels of RA (blue), PsA (green) and OA (orange) patients compared to total numbers of mDC (a), (b) and (c) and pDC (d), (e) and (f) respectively per ml of PB. CRP levels are calculated on a logarithmic scale and squares indicate individual samples. CRP correlation to PB DC numbers was analysed by Pearson Correlation. \* $p < 0.05$ .



**Figure 3.5 RA and PsA PB pDC and mDC have an immature phenotype and significantly decreased CD62L expression.**

Migration and maturation markers on CD1c<sup>+</sup>/CD11c<sup>+</sup> mDC purified from RA PB (n=5) and PsA PB (n=5) compared to healthy control PB (n=5) (a) and CD303<sup>+</sup>/CD123<sup>+</sup> pDC purified from RA PB (n=5) and PsA PB (n=5) compared to healthy control PB (n=5) (b). Values are calculated as the mean fluorescent intensity (MFI) fold of increase over the isotype control. The box and leaf plots depict median and interquartile range (50% of the data), flanked by whiskers indicating the extent of the data. \*p<0.05. Typical examples of FACS histograms analysing CD62L fluorescence intensity on pDC and mDC, compared to their respective controls, for one healthy donor PB sample and one RA PB sample are shown (c). Statistical analysis was carried out using Mann Whitney.



maturation marker CD83 and low to absent expression of CD40, CD80 and CD86 and the chemokine receptor CCR7. However, expression of the adhesion molecule CD62L (L-selectin) was significantly reduced on inflammatory arthritis derived mDC (RA  $p=0.0371$ , PsA  $p=0.0122$ ) and pDC (RA  $p=0.0367$ , PsA  $p=0.0373$ ) compared to healthy control PB mDC and pDC respectively (Fig. 3.5c). In order to account for inter-individual variability the FACS analysis results were calculated as the fold increase of the mean fluorescence intensity (MFI) over each individual isotype control, thus enabling comparative analysis across separate donors.

### **3.3.6 RA and PsA SF contain mDC and pDC.**

The foregoing data strongly suggest that DC subset distribution in inflammatory arthritis is abnormal and commensurate with either enhanced migration to the synovial compartment or reduced release from bone marrow. To investigate the former possibility, we chose to evaluate DC subsets freshly isolated from SF. To this end, we enumerated mDC and pDC in SF and matched PB obtained from 6 patients with RA and 6 patients with PsA. Magnetic sorting and flow cytometry revealed that whereas absolute mDC and pDC numbers varied greatly between individual patients, both subsets were consistently present in RA and PsA SF (Table 3.2). The ratio of mDC: pDC in PB was approximately 2:1 in both RA and PsA patients and was similar to that seen in OA patients and healthy controls (Fig. 3.6a). In contrast, this ratio was significantly increased in both RA (median 9.4:1,  $p=0.0082$ ) and PsA (median 6.05:1,  $p=0.0453$ ) SF (Fig 3.6b) compared with matched PB, suggesting enrichment of these populations in tissue.

### **3.3.7 SF mDC have a semi-mature phenotype but pDC are immature.**

To investigate the phenotype of mDC and pDC in SF, cells were isolated from a subset of RA SF ( $n=3$ ) and PsA SF ( $n=4$ ), as stated above, and analysed by FACS analysis using a range of cell surface markers. Isolated DC subsets were routinely >90% pure. Phenotype was determined by gating specifically on CD1c<sup>+</sup>/CD11c<sup>+</sup> cells (mDC) and CD303<sup>+</sup>/CD123<sup>+</sup> cells (pDC). Once again the FACS analysis results were interpreted as the fold increase of the MFI above each individual isotype control to allow comparative analysis. In both RA SF (Fig. 3.7a) and PsA SF (Fig. 3.7b) the phenotype of mDC was similar, with increased expression of CD40, CD80 (significant in PsA  $p=0.037$ ), CD83 and CD86 (significant in PsA,  $p=0.036$ ) in comparison to their RA and PsA PB counterparts respectively, indicating a semi-mature phenotype. In contrast, RA SF (Fig. 3.7c) and PsA

**Table 3.2.** Absolute number of cells in RA and PsA PB matched SF.

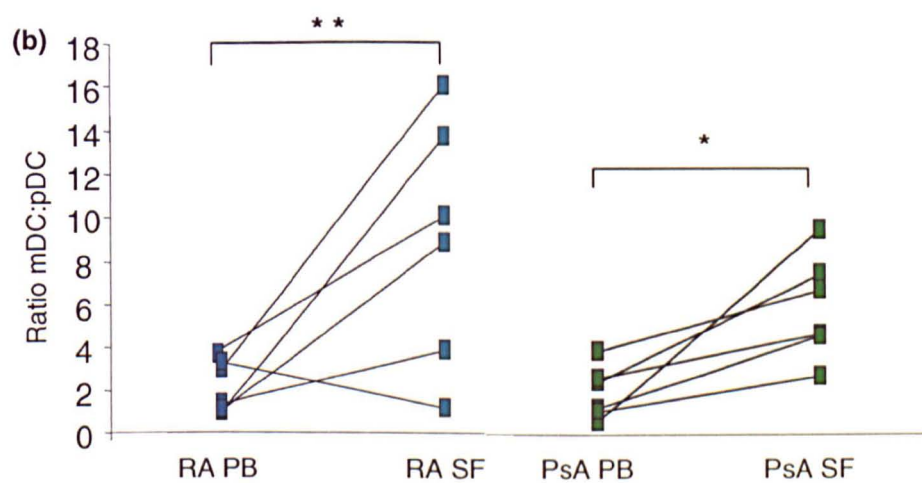
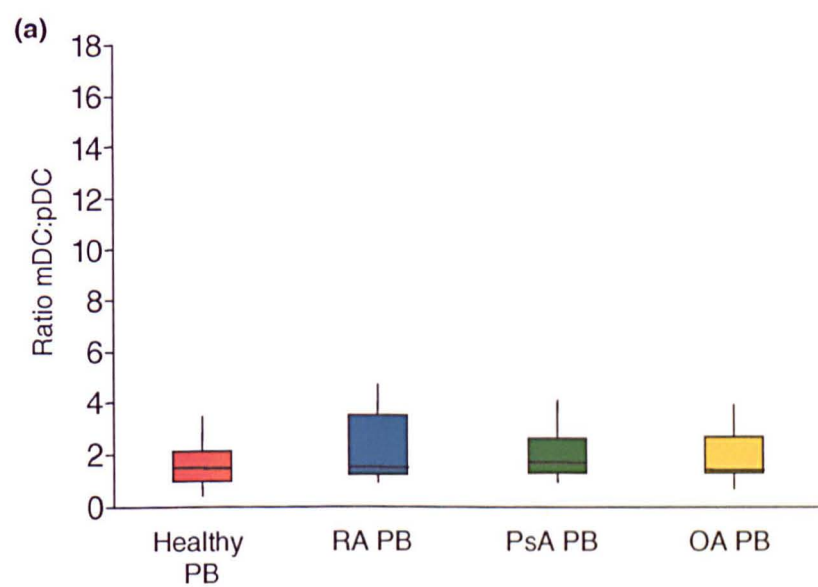
		WBC ( $\times 10^4/\text{ml}$ )	mDC ( $\times 10^4/\text{ml}$ )	pDC ( $\times 10^4/\text{ml}$ )
RA n=6	PB	652 $\pm$ 93.7	1.36 $\pm$ 0.641	0.582 $\pm$ 0.211
	SF	1020 $\pm$ 235	11.6 $\pm$ 3.12	3.24 $\pm$ 1.71
PsA n=6	PB	969 $\pm$ 240	1.39 $\pm$ 0.420	0.761 $\pm$ 0.220
	SF	2860 $\pm$ 351	37.5 $\pm$ 8.72	7.20 $\pm$ 2.23

Data are mean  $\pm$  SEM.

**Figure 3.6 mDC:pDC ratios in SF are significantly increased when compared to matched PB.**

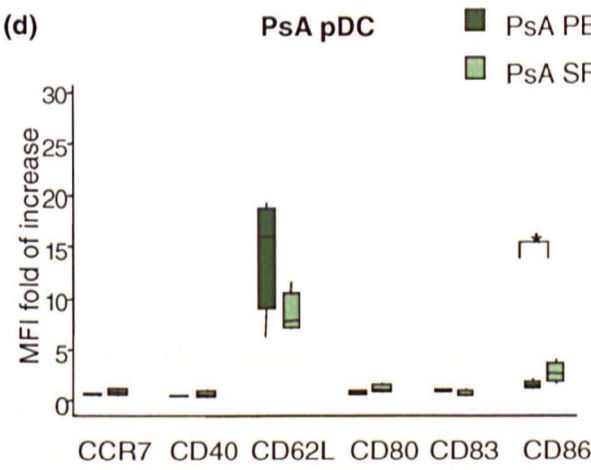
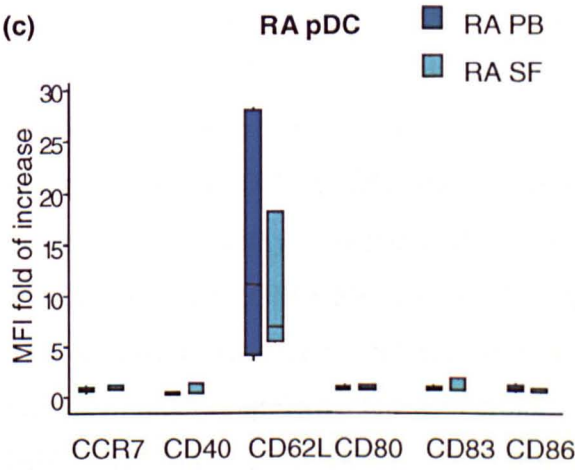
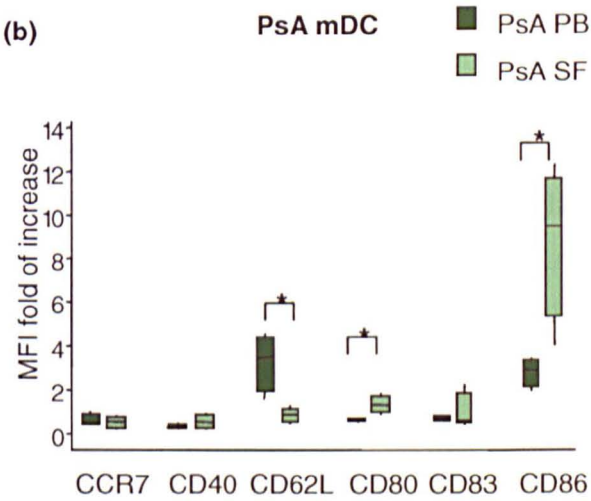
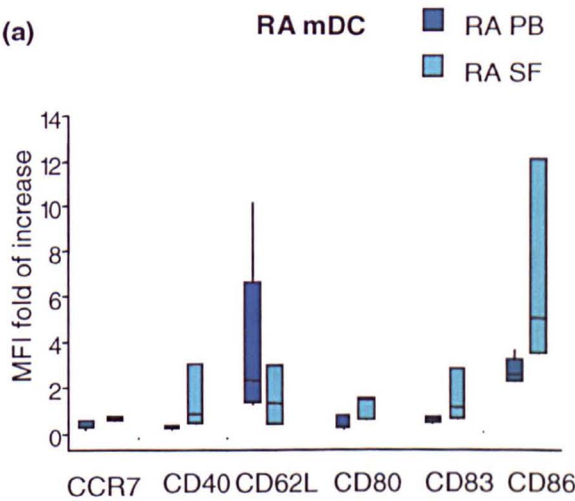
The ratio of mDC: pDC in PB from RA (dark blue, n=12), PsA (dark green, n=13) and OA (orange, n=11) subjects was not significantly altered compared to healthy control subjects (red, n=12) (a), median and interquartile range is shown. The ratio of mDC: pDC in SF from RA (n=6) and PsA (n=6) subjects was significantly increased compared to matched PB samples (b). Squares indicate individual matched samples. The ratio was calculated from total number of cells/ml. The scale is the same for both graphs to allow comparison. \*p<0.05, \*\*p<0.01. Statistical analysis was carried out using Mann Whitney.





**Figure 3.7 RA and PsA SF pDC have an immature phenotype but mDC have a semi-mature phenotype.**

Migration and maturation markers on CD1c<sup>+</sup>/CD11c<sup>+</sup> mDC purified from RASF (n=3) compared to RA PB (n=5) (4) (a) and PsA SF (n=3) compared to PsA PB (n=5) (b) and CD303<sup>+</sup>/CD123<sup>+</sup> pDC purified from RASF (n=3) compared to RA PB (n=5) (c) and PsA SF (n=3) compared to PsA PB (n=5) (d). Values are calculated as the mean fluorescent intensity (MFI) fold of increase above the isotype control. The box and leaf plots depict median and interquartile range (50% of the data), flanked by whiskers indicating the extent of the data. \*p<0.05. Statistical analysis was carried out using Mann Whitney.



SF (Fig. 3.7d) pDC displayed an immature phenotype, generally comparable to that of circulating PB pDC, with low to absent expression of CD40, CD80 and CD83. The only difference observed was an increase of CD86 expression on PsA SF pDC compared to PsA PB pDC ( $p=0.046$ ), although this was not observed on RA SF pDC. Since CD62L was significantly reduced compared with healthy volunteers but not absent, on mDC and pDC from RA and PsA PB, we also evaluated expression on SF derived cell subsets. CD62L was further decreased on mDC and pDC from both RA and PsA SF DC subsets compared to RA and PsA PB (significant in PsA SF mDC,  $p=0.049$ ).

The presence of relatively large numbers of immature pDC in SF was intriguing, since immature pDC characteristically produce high levels of IFN- $\alpha$  (591). The level of IFN- $\alpha$  expression in inflammatory synovial fluids has rarely been characterised, and if sought declared absent in previous publications (125). We therefore analysed SF from RA ( $n=18$ ), PsA ( $n=14$ ) and OA ( $n=7$ ) for the presence of IFN- $\alpha$  by ELISA (Fig. 3.8). By this methodology, IFN- $\alpha$  was detectable in many synovial fluids, derived not only from RA and PsA but also from a small number of OA SF. However, there were no significant differences when disease states were compared.

### **3.3.8 SF can differentiate monocytes into mDC**

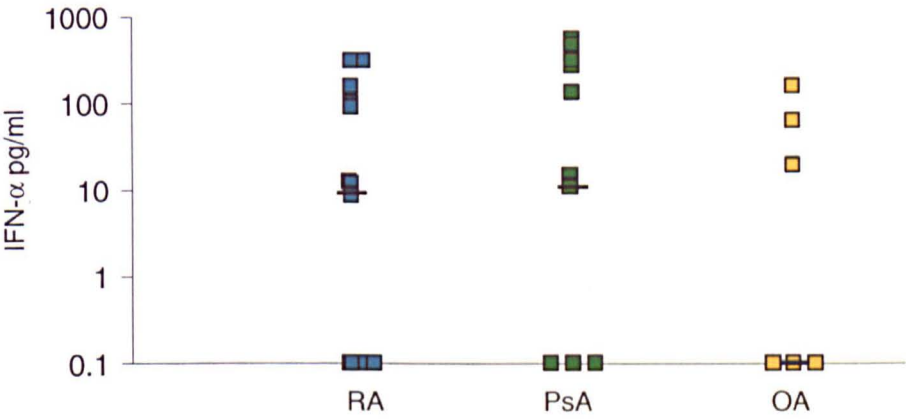
I next considered whether SF itself contained factors that drive mDC semi-maturation. It has previously been reported that SLE serum is sufficient to differentiate monocytes into monocyte-derived DC (moDC) due to its high IFN- $\alpha$  content (498). Monocytes were purified from healthy volunteer PB and cultured in granulocyte-macrophage-colony stimulating factor (GM-CSF) and IL-4 supplemented media as a positive control (Fig. 3.9b) or media alone as a negative control (Fig. 3.9a). The remaining monocytes were cultured in media supplemented with either 10% cell free SLE SF (Fig. 3.9c), 10% cell free OASF (Fig. 3.9d), 10% cell free PsA SF (Fig. 3.9e) or 10% cell free RA SF (Fig. 3.9f). All SF was sterile filtered. Light microscopic images of morphological alterations and FACS analysis of cell phenotype were used to assess cellular conversion.

After 5 days of culture the monocytes in media alone did not have good viability and showed no proliferative capacity. The monocytes cultured in SF supplemented media were viable and proliferating and morphologically, were comparable to moDC driven by the

**Figure 3.8 RA, PsA and OA SF contain IFN- $\alpha$ .**

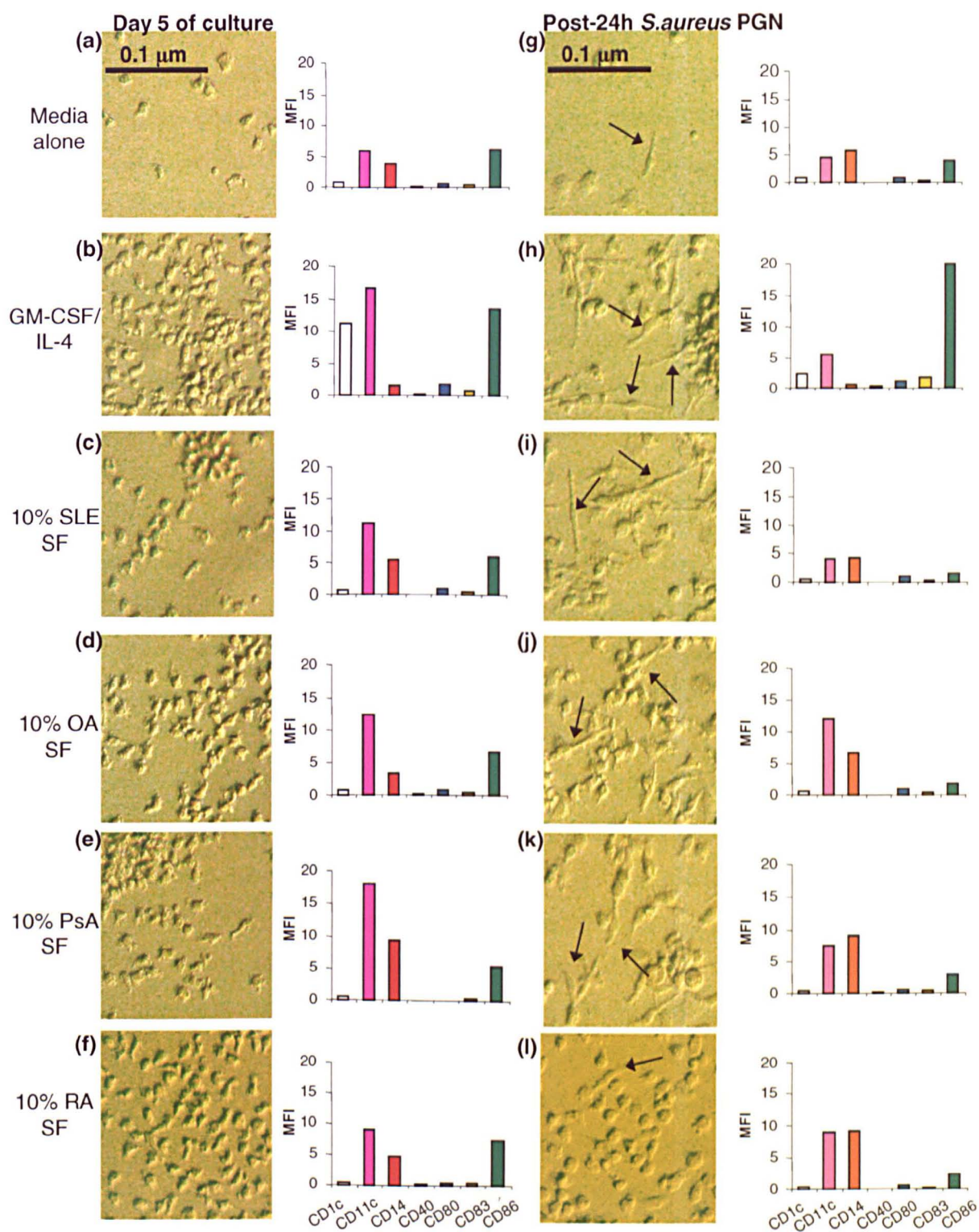
The level of IFN- $\alpha$  in SF from RA (n=18), PsA (n=15) and OA (n=8) was measured by ELISA. Squares indicate individual samples expressed on a log scale. - = median.

Synovial Fluid



**Figure 3.9 The SF mediated conversion of PB monocytes to mDC.**

Light microscopic phase contrast images and phenotype pre and post 24 h incubation with *S.aureus* PGN (10µg/ml) of monocytes purified from healthy PB and cultured in GM-CSF/IL-4 (**a** and **g**), media alone (**b** and **h**), 10% SLE SF (**c** and **i**), 10% OA SF (**d** and **j**), 10% PsA SF (**e** and **k**) or 10% RA SF (**f** and **l**). Arrows indicate mature DC with typical dendrite extensions.





traditional culture system using GM-CSF and IL-4. FACS analysis of cellular phenotype indicated that all SF supplemented media cultures had up-regulated CD11c expression to comparable levels with that driven by GM-CSF and IL-4, consistent with moDC conversion. Expression of CD1c was also upregulated on SF supplemented cultures, but not to levels as high as that observed in GM-CSF/IL-4 supplemented cultures. Furthermore, CD14 persisted on the cell surface of SF cultured cells at levels higher than that observed on GM-CSF/IL-4 cultured cells. Expression of CD40, CD80 and CD83 was low to absent, while CD86 was expressed on cells consistent with a predominantly immature phenotype.

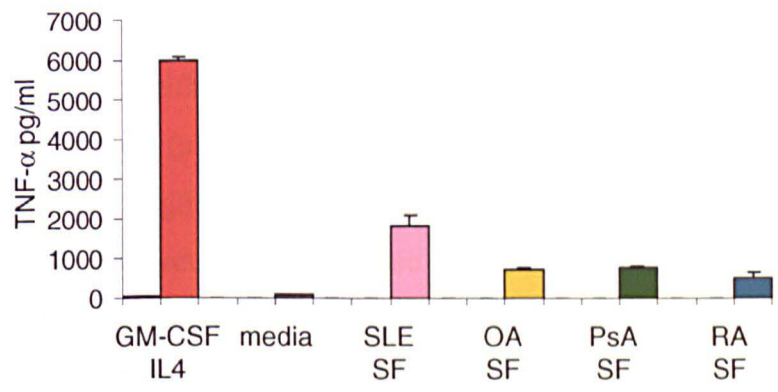
### **3.3.9 SF inhibits TLR2 stimulated TNF- $\alpha$ release from moDC**

A number of classical animal models of arthritis, such as streptococcal cell wall induced arthritis, are dependent upon the engagement of the innate immune system via TLR signalling. TLR2 deficient animals display significantly reduced severity of arthritis (592). Moreover, bacterial peptidoglycans (PGN) capable of acting as agonists to TLR2, have been detected in the joints of patients with inflammatory arthropathies (593) and APC (otherwise poorly characterised) containing PGN have been identified in inflamed synovium (594). It is now recognised that DC are activated via TLRs (491). Thus TLR induced phenotypic changes and cytokine production were evaluated in order to assess the functional capacity of SF converted moDC using the TLR2 agonist *S.aureus* PGN (10 $\mu$ g/ml).

Upon exposure to PGN, GM-CSF/IL-4 cultured moDC matured both morphologically, phenotypically and functionally as expected. Dendrite expression and up-regulation of CD80, CD83 and CD86 and slight down-regulation of CD1c and CD11c (Fig. 3.9g) were observed and release of TNF- $\alpha$  to agonist was increased (Fig. 3.10). Media cultured monocytes did not alter dramatically (Fig. 3.9h), nor did they release TNF- $\alpha$ . Surprisingly, while many moDC in both SLE and OA SF culture matured morphologically with evident dendritic expression, CD80, CD83 and CD86 were not upregulated on the cell surface, indicating aberrant maturation (Fig. 3.9i and 3.9j respectively). Consistent with this observation, SLE and OA SF cultured moDC were capable of only low level TNF- $\alpha$  release (Fig. 3.10). Furthermore, only a small percentage of PsA and RA SF cultured moDC attained mature morphology, but like SLE and OA SF cultured moDC, did not

**Figure 3.10 SF cultured moDC release only low levels of TNF- $\alpha$ .**

TNF- $\alpha$  levels in GM-CSF/IL-4 cultured moDC (red), media cultured monocytes (purple), SLE SF cultured moDC (pink), OA SF cultured moDC (orange), PsA SF cultured moDC (green) and RA SF cultured moDC were analysed by ELISA following 24 h stimulation with (filled bars) or without (levels too low to appear) *S.aureus* PGN (10  $\mu$ g/ml) and expressed as pg/ml. One representative example from each of thrice replicated experiments is shown.



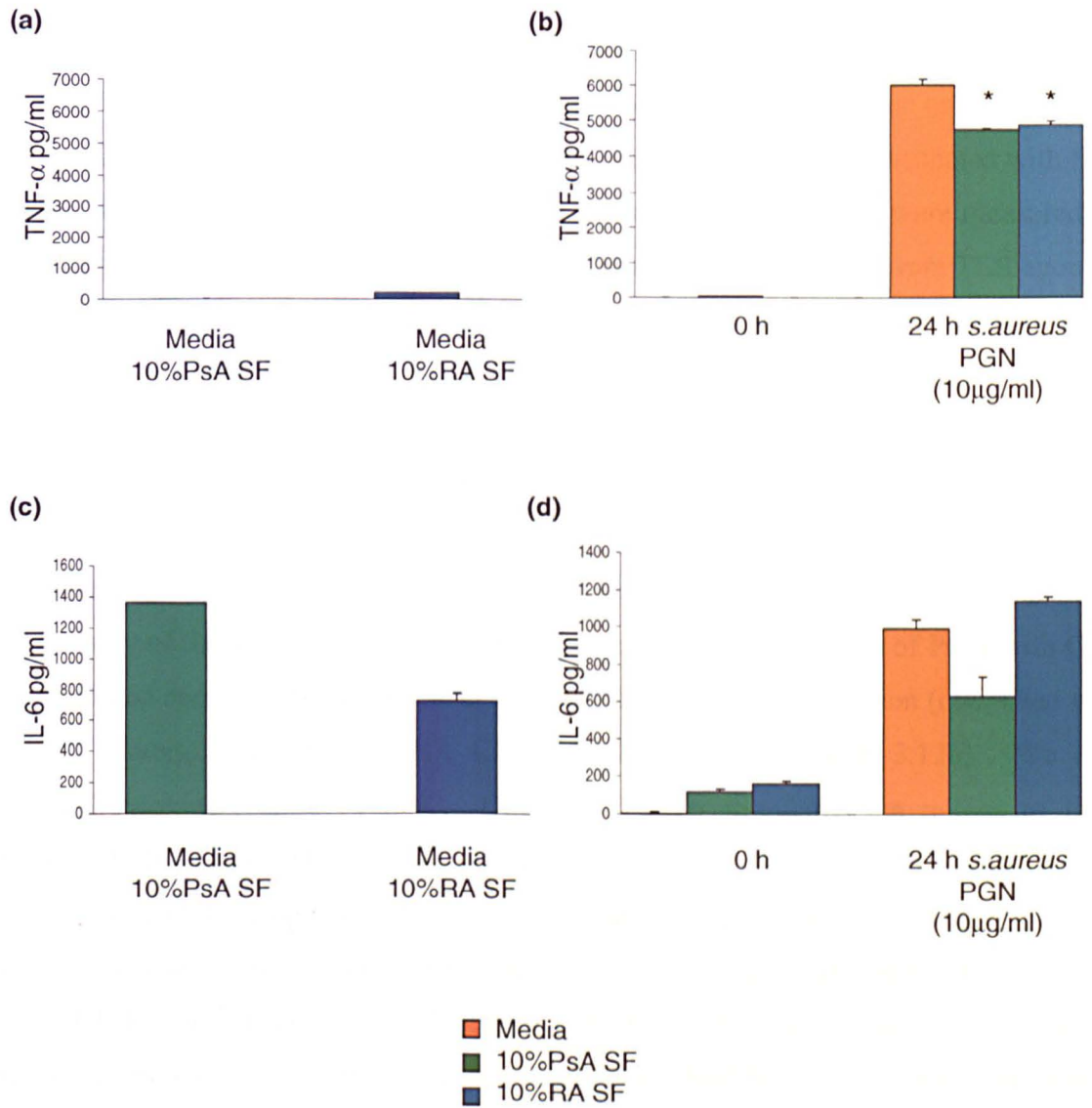
attain a mature phenotype (Fig. 3.9k and 3.9l respectively). Again, consistent with this observation, PsA and RA SF cultured moDC were capable of only low levels of TNF- $\alpha$  release following TLR2 stimulation (Fig. 3.10). Together these data indicate a number of possibilities. Firstly, whereas SF from RA, PsA and OA is sufficient to drive conversion of monocytes to moDC this process is incomplete resulting in the presence of intrinsic cellular defects. Alternatively, factors in the SF itself may directly inhibit the function of moDC.

In order to assess the latter possibility, monocytes were cultured into moDC using GM-CSF/IL-4 and at day 5 of culture were incubated in the presence or absence of either 10% cell free RA SF, or 10% cell free PsA SF. The SF in both cases was composed of a pooled cocktail of SF from 5 separate donors. After 24 hours, *S. aureus* PGN (10 $\mu$ g/ml) was added to wells and incubated for a further 24 hours before supernatants were analysed for TNF- $\alpha$  levels by ELISA. All cultures released TNF- $\alpha$  in response to TLR2 stimulation, however cells in the presence of either RA SF or PsA SF released significantly less TNF- $\alpha$  than those cells in media alone ( $p=0.016$  and  $p=0.024$  respectively) (Fig. 3.11b). Both RA SF and PsA SF contained negligible levels of TNF- $\alpha$  a priori (Fig. 3.11a). The data indicate that factors in SF, irrespective of diagnostic criteria, may either partially inhibit TLR2 mediated activation or may inhibit TNF- $\alpha$  release through anti-inflammatory activity. Alternatively soluble (s) TNF-receptor 1 (TNFR1) in SF may bind to some of the TNF- $\alpha$  released thus resulting in a lower measurement on ELISA. To assess this possibility IL-6 release was also measured. Both RA SF and PsA SF contained substantial levels of IL-6 (Fig. 3.11c), thus culture supernatants were calculated as the concentration of IL-6 above the background. Analysis revealed that both RA and PsA SF were sufficient in themselves to elicit IL-6 release from moDC (Fig. 3.11d). IL-6 levels were further increased after TLR2 activation in all cultures, indicating that SF is neither inhibiting TLR2 activation nor moDC activity. The data are instead consistent with sTNFR1 present in SF binding TNF- $\alpha$  released by DC. However, as background IL-6 levels were high, additional experiments analysing intracellular mechanisms of activation such as NF- $\kappa$ B phosphorylation and transcriptional IL-6 mRNA are required in order to investigate this finding further.

**Figure 3.11 SF inhibits TNF- $\alpha$  release from GM-CSF/IL-4 cultured moDC.**

TNF- $\alpha$  (b) and IL-6 (d) levels in GM-CSF/IL-4 monocyte differentiated moDC incubated for 24 h in media alone (red), 10%PsA SF (green) or 10%RA SF (blue) were analysed by ELISA pre and post 24 h stimulation with *S.aureus* PGN (10  $\mu$ g/ml) and expressed as pg/ml. The background levels of TNF- $\alpha$  (a) and IL-6 (c) in cell free media containing 10%PsA SF or 10%RA SF alone was also analysed. Levels of TNF- $\alpha$  and IL-6 were corrected for the background levels as measured in the SF alone.

\*p<0.05 Statistical analysis was carried out using Student's T test.



### 3.3.10 SF mDC and pDC mature and release cytokines upon TLR stimulation.

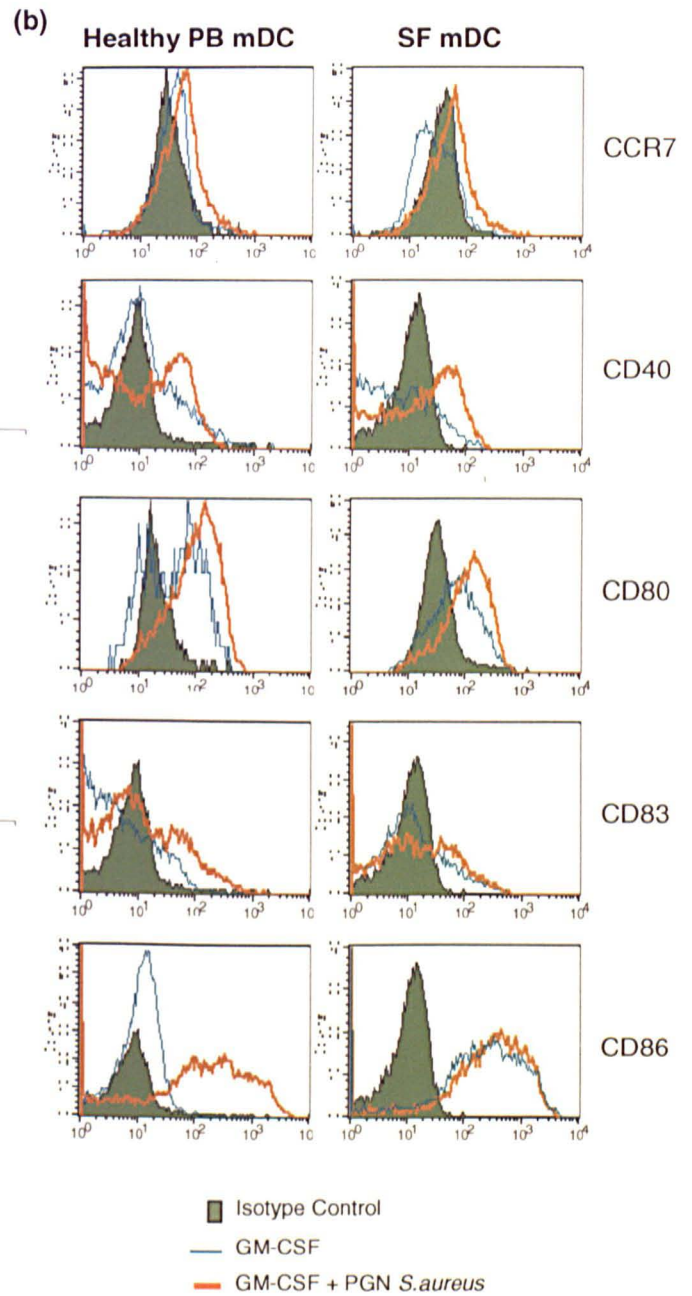
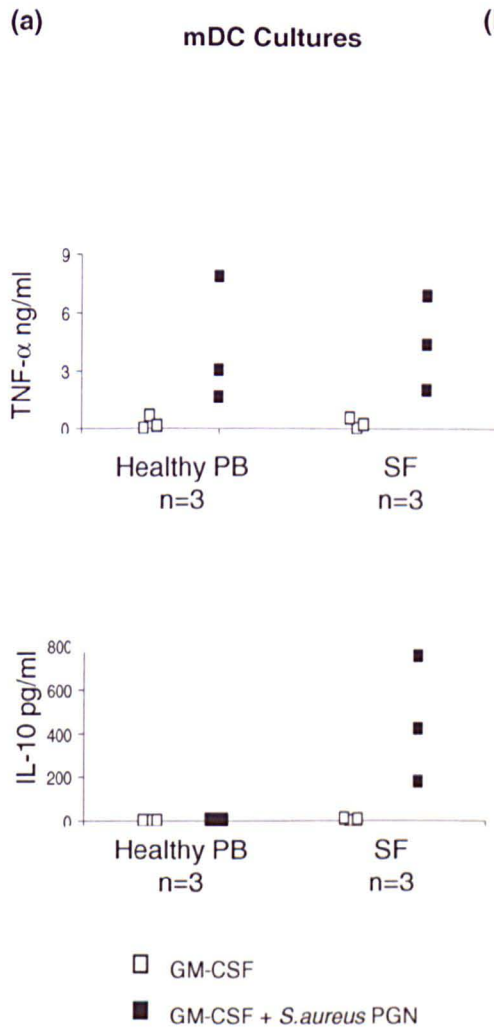
In order to determine if DC subsets within SF have an intrinsic defect preventing normal function both subsets were purified from SF and response to TLR stimulation analysed. It is recognised that DC are activated via TLRs (491). Thus TLR induced cytokine production was evaluated in disease compared with healthy PB derived DC in order to determine if DC subsets within SF have an intrinsic defect.

We purified mDC and pDC as previously described and evaluated addition of the TLR2 agonist *S.aureus* PGN (for mDC) or the TLR9 agonist CpG ODN 2216 (for pDC) over 24h. For control purposes, mDC were also incubated with GM-CSF alone or the irrelevant TLR agonist CpG ODN 2216 (mDC do not express TLR9). pDC were incubated with IL-3 alone or with control ODN 2216c. Phenotypic changes in DC subsets were measured by FACS analysis and cytokine release by multiplex assay or ELISA. Irrelevant TLR agonists did not induce cytokine production in any culture conditions tested, nor had they any effect on *in vitro* maturation of DC subsets (data not shown). Distinct patterns of cytokine production emerged on comparison of PB versus SF derived mDC and pDC. In PGN-stimulated cultures mDC from both PB and SF produced comparable levels of TNF- $\alpha$  (Fig. 3.12a). In contrast, SF mDC produced considerably higher levels of IL-10 following PGN stimulation than did PB comparators. IL-12p70, IL-2 and IL-4 levels were below the limit of sensitivity of the assay in cultures. Furthermore, coincident addition of PGN with GM-CSF produced considerably higher levels of maturation marker expression (compared with GM-CSF alone), particularly CCR7, CD40, CD80 and CD83 (Fig. 3.12b). We next examined cytokine release by pDC. CpG ODN 2216 stimulated the release of large amounts of IFN- $\alpha$  and TNF- $\alpha$ , from PB and SF derived DC. However only SF pDC produced IL-10 following CpG ODN 2216 stimulation (Fig. 3.13a). Neither IL-2 nor IL-4 release was detected under these conditions. Upon phenotypic analysis upregulation of CCR7, CD80 and CD83 (Fig. 3.13b) after CpG ODN 2216 stimulation was observed. Finally, we noted that growth factor alone, namely GM-CSF or IL-3, was sufficient to promote maturation marker expression but did not induce cytokine production. Together these data indicate that SF DC subsets retain the capacity to upregulate maturation markers and respond to TLR agonists and that the latter are necessary to promote optimal maturation and cytokine production. They further show no intrinsic specific maturation failure or block.

**Figure 3.12 SF mDC mature and release TNF- $\alpha$  at comparable levels to healthy PB mDC, but only SF mDC release IL-10.**

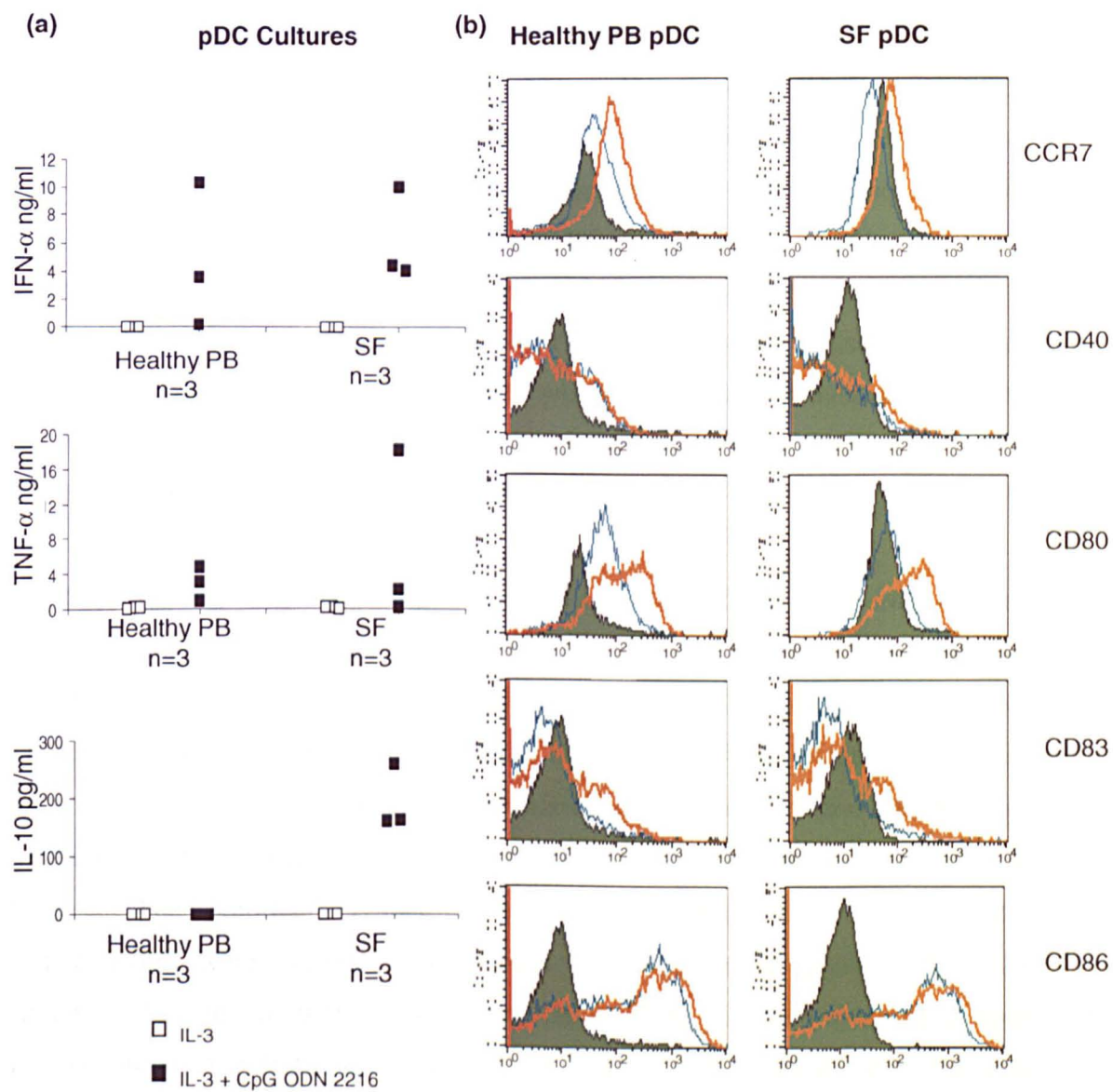
Cytokine release and maturation status of mDC purified from healthy control PB (n=3) or inflammatory arthritis SF (RA=2, PsA=1) were assessed after 24 h incubation with GM-CSF supplemented media alone or containing *S.aureus* PGN (10  $\mu$ g/ml). The concentrations of TNF- $\alpha$  and IL-10 (a) in supernatant from the cultures was determined by multiplex analysis and expressed as release per  $5 \times 10^4$  cells. Squares indicate individual donors. Maturation and co-stimulation marker expression was determined by flow cytometry and one representative example from each of the healthy control PB and inflammatory arthritis SF groups are shown (b).





**Figure 3.13 SF pDC mature and release IFN- $\alpha$  and TNF- $\alpha$  at comparable levels to healthy control PB pDC, but only SF pDC release IL-10.**

Cytokine release and maturation status of pDC purified from healthy control PB (n=3) and inflammatory arthritis SF (RA=2, PsA=1) were assessed after 24 h incubation with IL-3 supplemented media alone or containing the TLR9 agonist CpG ODN 2216 (3.2  $\mu$ g/ml). The concentrations of IFN- $\alpha$ , TNF- $\alpha$  and IL-10 (a) in supernatant from cultures was determined by multiplex and ELISA analysis and expressed as release per  $5 \times 10^4$  cells. Squares indicate individual donors. Maturation and co-stimulation marker expression was determined by flow cytometry and one representative example from each of the healthy



### 3.4 Discussion

The relative role of innate and adaptive components of the host immune response to inflammatory synovitis is of considerable current interest. DC comprise a heterogeneous network of professional antigen presenting cells, capable not only of presenting antigen and inducing subsequent adaptive immune responses, but also of sensing the inflammatory environment and contributing to the cytokine milieu therein. Whilst implicated in the pathogenesis of RA and PsA (273, 294-296, 550, 552), prior analysis of DC subsets has been hampered by a lack of specific DC markers and reliable quantitation methodologies. This study has utilised the pDC specific CD303 and the mDC marker CD1c within a sensitive assay system to provide enumeration and phenotypic analysis of the DC subsets in PB and SF from PsA and RA patients. Thereafter we have utilised novel markers to purify DC subpopulations and perform a functional evaluation of their activation potential *ex vivo*. Finally we have for the first time attempted a phenotypic description of DC subsets in PsA and compared them with those in RA.

The first observation was reduction in circulating PB pDC and mDC populations in RA and pDC populations in PsA. That this was not observed in prior investigation probably reflects less sensitive methodologies (125, 595) and identification of subsets based upon CD123 expression, which is also expressed by basophils (596), hematopoietic progenitor cells (597), endothelial cells (598), monocytes, eosinophils and small subsets of lymphocytes (599). The observed decrease in DC numbers is not due to steroid therapy, which has been reported to reduce DC numbers (600) and function (601), as patients on corticosteroids were excluded. Decreased PB DC numbers most likely reflect DC migration from the circulation and accumulation to the inflamed synovial compartment. This is consistent with previous studies (106, 125, 595), and with our own data indicating comparatively high numbers of mDC and pDC in SF effusions from both PsA and RA. In addition, SF DC subset expression of CD62L (normally cleaved from the cell surface after leukocyte tethering and rolling across high endothelial venules (602) was lower on both PsA and RA SF compared to the peripheral blood compartment further indicative of migration. The trend towards decreased PB pDC numbers in the OA patients, although not significant, may be indicative of altered DC migration toward the synovial compartment. OA is associated with synovial hypertrophy, hyperplasia, local inflammation and recurrent

effusions (603). Commensurate with this hypothesis, we have identified a statistically significant inverse correlation between acute phase response as a surrogate for disease activity and circulating PB DC subsets.

Other factors however may operate in regulating detectable DC numbers, particularly within the bone marrow in which increasingly it is recognised that functional maturation defects may exist for leukocyte lineages (604). For example, reduced pDC numbers in circulating PB could reflect elevated TNF- $\alpha$  expression (605, 606): TNF- $\alpha$  is known to inhibit fms-like tyrosine kinase 3-ligand (FLT-3L) driven differentiation of pDC from bone marrow progenitors (591, 607). Future studies will be required to establish the circulating half life and tissue destination of DC subsets in the context of inflammatory arthropathies, and indeed of the effect of cytokine blockade on such parameters. It should also be noted that reduced circulating DC subsets have been demonstrated in several other inflammatory disorders including SLE (588) and chronic active hepatitis (608) and regulatory pathways in such diseases may also be informative in elucidating DC function in synovitis.

A further novel observation was reduced CD62L expression on both DC subsets in PsA and RA PB. Previous reports of reduced CD62L expression on RA PB CD3<sup>+</sup> and CD19<sup>+</sup> lymphocytes, monocytes and granulocytes (609) have implicated immunosuppressive therapies including methotrexate (610) and a similar mechanism could explain our observations. However down-regulation of adhesion molecules such as CD62L is necessary for the release of CD34<sup>+</sup> progenitors to the PB (611), and it is possible that a CD62L<sup>lo</sup> PB DC population may indicate altered recirculation, or modified release of bone marrow DC precursors. In addition, after migration across HEV, CD62L is rapidly shed from the cell surface via proteolytic cleavage producing a soluble (s) CD62L isoform, a process considered essential in order to limit the inflammatory process. A number of groups have reported elevated levels of sCD62L in RA serum (612, 613), consistent with intact CD62L mediated leukocyte migration. Certainly, the accumulation of pDC and mDC in the SF indicates that migration towards the inflamed compartment is not impeded. However, as DC comprise fewer than 1% of all leukocytes it is doubtful that migration of these cells alone would alter serum sCD62L significantly.

SF purified pDC were recently reported to exhibit a relatively immature phenotype given their inflammatory environment (125, 595). Similar observations have been made for

mDC (106, 535). We now confirm these observations in RA and extend the data to include PsA derived cells indicating that the inflamed synovial compartment *per se* can sustain immature DC capable, by inference, of antigen sensing, capture and ultimately presentation. Both mDC and pDC upon removal from the synovial fluid were capable of increased maturation markers expression and provided with TLR agonist activation were capable of cytokine release. There does not therefore appear to be an intrinsic functional defect of DCs. Whereas TLR2 or TLR9 stimulation was not sufficient to induce substantial IL-10 release from healthy PB mDC and pDC, it was sufficient for IL-10 release from PsA and RA SF purified cells. The functional implications of this observation are unclear and require further examination to include other TLR agonists and DC activation conditions.

We wished to examine the cross regulation of mDC and pDC subsets in the context of inflammatory synovitis. That said the technical demands of isolating primary DC from an inflamed compartment (particularly pDC) are not insignificant and placed limitations on the extent of functional analyses that were feasible. Very few groups have thus far attempted formal functional evaluation of purified DC subsets from any inflamed tissue. Other groups have utilised monocyte derived DC (moDC), generated from RA PB, to assess by inference DC function in arthritis. In particular these analyses have suggested that DC in inflammatory arthritis are hyper-responsive to TLR2 and TLR4 stimulation, releasing more IL-1, IL-6, TNF- $\alpha$  and IL-10 (614, 615). However, Iwahashi et al have reported that not only are CD16<sup>+</sup> monocytes elevated in peripheral circulation of patients with RA, but they also express higher levels of TLR2 than CD16<sup>-</sup> monocytes producing increased amounts of IL-1, IL-6, TNF- $\alpha$  and IL-8 in response to TLR2 stimulation (616). Thus it can be argued that the approach utilised in the earlier studies, although pragmatic, was inherently flawed by the use of monocytes to infer 'classical' DC function. In addition 5 days of culture in growth factors are required to drive monocyte differentiation into moDC, thus culture artefact cannot be discounted in these studies. Thus in order to gain insight into DC function in disease we chose to purify DC directly as, although this presented major difficulties in obtaining sufficient numbers, we believed it was necessary in order to identify differences in DC function and discount starting cell population or culture artefact. Our approach combines a broad but pragmatic approach to start to examine DC function *ex vivo*. Future technical refinements will be required to perform the

desired extensive *in vitro* analyses of DC function that will more comprehensively address their functional role.

These are the first data to investigate purified DC subsets in PsA patients. Prior studies have identified pDC in cutaneous psoriasis and increased type I IFN expression in psoriatic plaques after application of a TLR agonist (617). Numerous data indicate a role of T cells in PsA pathogenesis (233) and as such determining the functional role of both pDC and mDC in PsA synovium should be informative. The present study provides the essential pre-requisite for such studies in determining the presence, enumeration and function of each subset in PB and SF.

IFN- $\alpha$  was detected in SF in both RA and PsA patients. The role for this cytokine in synovial pathogenesis is indicated by numerous reports linking IFN- $\alpha$  therapy to induction of RA and PsA in patients with no pre-existing clinical arthritis (545, 618, 619). Moreover, IFN- $\alpha$  is sufficient to drive monocyte differentiation to mDC in SLE patients (518). The significantly increased ratio of mDC:pDC identified in SF, may indicate *in situ* differentiation of monocytes shed from the synovial lining layer to mDC, driven in part by IFN- $\alpha$  in fluid. It is therefore intriguing to consider a local cytokine mediated feedback loop whereby pDC IFN- $\alpha$  release promotes local mDC maturation. Consistent with this we observed that PsA and RA synovial fluid is capable of inducing differentiation of DC from PB derived monocytes (moDC). Unexpectedly however, SF differentiated moDC released only low levels of TNF- $\alpha$  in response to TLR2 stimulation as compared with GM-CSF/IL-4 differentiated moDC and this occurred irrespective of SF origin (RA/PsA/OA/SLE). Considering that both mDC and pDC purified directly from SF responded normally to TLR stimulation, indicating no intrinsic defect, it is likely that a soluble factor present in SF inhibited either the TLR stimulation or the release of TNF- $\alpha$ . It has been proposed that the intracellular signalling initiated by a major regulator of DC function, the inhibitory Fc gamma receptor IIb (Fc $\gamma$ RIIb), is defective in RA thus enabling chronic inflammation (620). Since Fc $\gamma$ RIIb stimulating immune complexes are abundant in SF, the decreased TNF- $\alpha$  released following TLR2 stimulation may have resulted from triggering of the (presumably) intact inhibitory Fc $\gamma$ RIIb pathway in moDC generated from healthy volunteer PB. In order to assess this possibility in future, analysis of this experiment using moDC generated from RA and PsA PB is necessary.

While only mature DC are able to induce the activation of T cells and the differentiation of B-cells into antibody-producing plasma cells, immature DC are able to capture antigen at picomolar and nanomolar concentrations (446). In the steady state, presentation of these self-antigens should sustain tolerance. However the presentation of these antigens within an inflammatory context, such as the inflamed synovial compartment in susceptible individuals, may lead to chronic inflammation and autoimmune disease. SF apparently contains a pool of immature DC subsets of both mDC and pDC lineage. It is likely that SF DC are not only crucial for (auto) antigen capture, but thereafter migrate into the synovial membrane leading to perpetuation, and perhaps initiation (neoepitopes) of autoimmunity. In order to assess this possibility, it was necessary to analyse the phenotype and distribution, particularly in relation to other leukocytes, of mDC and pDC *in situ* in inflamed synovial membrane. The results of these analyses will be discussed in Chapter 4.



## **Chapter 4: Analysis of the localisation, maturation and cytokine profile of plasmacytoid dendritic cells and myeloid dendritic cells in inflammatory synovitis**

## 4.1 Introduction

A characteristic of the inflamed synovium in RA and PsA is the rapid influx of mononuclear cells including CD4<sup>+</sup> and CD8<sup>+</sup> T cells, plasma cells, B cells, NK cells, monocytes, mast cells and DC (101-105, 107, 123-125). In 40-50% of PsA and RA cases cellular infiltration progresses towards the development of lymphoid-like structures, with HLA-DR expressing CD4<sup>+</sup> T cells localised to aggregates in close association with MHC expressing macrophages and DC, as well as B cells. The synovium is therefore generally considered an ectopic lymphoid organ, even though germinal centre formation occurs in as few as 5-23% of cases (132). Moreover, the lack of classical lymphoid topographical segregation in the majority of cases may enhance disease pathology, particularly with respect to the potential for *in situ* antigen presentation.

DC are the most potent professional APC, critical not only to the initiation and regulation of adaptive immunity but also the maintenance of both central and peripheral tolerance. As such, DC have been implicated in the initiation and perpetuation of chronic autoimmune disease through the abolition of self-tolerance and subsequent emergence of self-reactive lymphocytes. Significantly, it has recently been shown that the aberrant accumulation of DC in tissue, but not of T cells or B cells, is sufficient in itself to induce symptoms of autoimmunity including the production of antinuclear antibodies (621). Furthermore, DC infiltration to the inflamed synovial compartment occurs early in disease pathology (273).

A number of previous immunohistochemical analyses of (predominantly) RA synovium sought to characterise the presence and nature of (myeloid) DC infiltration. Immature CD1a<sup>+</sup> mDC have been identified in the RA synovial lining layer, while mature MHC I<sup>high</sup>/MHC II<sup>high</sup>/DC-lysosome-associated membrane protein (LAMP)<sup>+</sup>/CD83<sup>+</sup> cells have been localised predominantly to perivascular regions and lymphocyte aggregates (534). In addition, differentiated mDC expressing nuclear RelB as well as co-stimulatory molecules, both of which are associated with active APC function, have been identified in RA SM and this is consistent with hypotheses of DC driven autoreactive T cell expansion (273). However technical restrictions arising from a lack of specific DC surface markers, mean that past studies have been limited as they provided no real marker to distinguish mDC

from SM infiltrating macrophages, which also express such markers as CD83. Moreover, and in consequence, there is poor understanding of how DC phenotype differs within and between inflammatory arthropathies.

The origin of SM localised DC is unclear, although it has been suggested that immature SF DC may migrate towards CCL19/21 rich lymphocyte aggregates where they undergo local maturation. This hypothesis is supported by the apparent DC maturation 'gradient' described by prior studies identifying immature CCR6<sup>+</sup> mDC in CCL20 rich areas of the synovial lining layer and mature DC localised to ectopic lymphoid-like structures in the synovium (133). Alternative theories suggest a model whereby over-expression of precursor DC chemoattractants CCL2, CCL5 and CCL20, coupled with TNF- $\alpha$  driven up-regulation of adhesion molecules induces the transendothelial migration of PB DC direct to the synovium. According to this model, monocyte shedding to the SF and *in situ* differentiation to moDC, driven by the presence of multiple growth and differentiation factors including GM-CSF, TNF- $\alpha$  and IL-1, may account for SF DC (538). However, this model does not account for the presence of immature pDC in SF. Indeed, almost nothing is known of the infiltration or phenotype of pDC to the arthritic synovium.

A number of reports have recently identified pDC in the target tissue of numerous inflammatory disorders including contact dermatitis, psoriasis vulgaris and systemic lupus erythematosus (SLE) (437, 622). Furthermore, a critical role has been attributed to pDC in the aetiopathology of SLE and is associated with excessive IFN- $\alpha$  production (623). It is of note then that a role for this cytokine in synovial pathogenesis is indicated by numerous reports linking IFN- $\alpha$  therapy to induction of RA and PsA in patients with no pre-existing clinical arthritis (543-549). Critically, IFN- $\alpha$  is increasingly considered important to the pathology of psoriasis, a defining extra-articular feature of PsA (437). Clearly then, resolution of the pDC profile in inflamed tissue is required, particularly in the context of cytokine production as pDC are the primary type I IFN producing cells in the body.

Cytokines are pivotal players in inflammatory synovitis, as evidenced by the therapeutic success of TNF- $\alpha$  blockade. However many patients do not respond to therapy, and in those that do, long-term remission is rare. Thus in the treatment of RA, the critical challenge now is to uncover the underlying immunological processes responsible for its generation and perpetuation, such that peripheral tolerance can be re-established and

therapeutic remission can be maintained. To this end it is vital to understand what cytokines are produced by those cells most involved in the maintenance of self-tolerance, namely DC. Indeed, detailed analysis of cytokine expression by DC in the context of human inflammatory disease has, to our knowledge, been performed by only one other group (125).

## 4.2 Aims and Objectives

As described in Chapter 3, we have identified an increase in the synovial fluid, but decrease in the blood, of both pDC and mDC from RA and PsA patients. Considering the recent advances in identifying DC subsets by use of specific markers, it was therefore the aim of this chapter to undertake a comparative analysis of pDC and mDC in synovium from RA and PsA patients in order to address whether the reduction in PB DC subsets could be accounted for solely by accumulation to the SF, or whether these subsets also migrated to the SM. In addition, it was aimed to provide a more comprehensive investigation of the functional phenotype, including maturation status and cytokine profile, of mDC and pDC than has previously been attempted.

Specifically, it was planned to address:

- Whether pDC and mDC could be detected in the SM of patients with RA, PsA or OA using immunohistochemical techniques.
- The maturation profile of RA, PsA and OA SM infiltrating pDC and mDC as assessed by CD83 and DC-LAMP expression.
- Whether pDC and mDC within synovium could be localised to lymphocyte aggregates, specifically with reference to CD3<sup>+</sup> and CD8<sup>+</sup> T cells.
- Whether pDC and mDC display the same or different cytokine profiles in SM, by analysis of IL-12p70, IL-23p19, IL-15, IL-18, IFN- $\alpha$  and IFN- $\beta$ .
- Finally, whether there exist any identifiable and specific differences in the number and phenotype of pDC and mDC from RA SM in comparison to PsA SM.

## 4.3 RESULTS

### 4.3.1 Patients and tissue samples

Inflammatory arthritis patients attending the GRI and the Academic Medical Centre (AMC) in Amsterdam fulfilled the American College of Rheumatology criteria for RA (Arnett), or met diagnostic criteria for PsA as previously described. All patients and healthy donors gave informed consent and the study protocol was approved by the GRI Ethical Committee and the AMC Medical Ethics Committee. Twenty RA patients, ten osteoarthritis (OA) patients and nine psoriatic arthritis (PsA) patients were included in this study (Table 4.1). Patients were receiving disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate (no more than 15 mg/week), hydroxychloroquine, or sulfasalazine, of stable dose for at least two months. Non-steroidal anti-inflammatory drugs (NSAIDs) were allowed, provided that the dose and frequency had been stable for 30 days. Small-bore arthroscopy was performed under local anesthesia and synovial tissue samples were obtained from multiple sites in the joint using 2-mm grasping forceps (Storz). Synovial tissue matched skin biopsies were taken from one patient with PsA. Biopsy samples were collected and snap-frozen in TissueTek OCT (Miles Elkhart). Frozen blocks were stored in liquid nitrogen until sectioned for staining. Sections (5  $\mu$ m) were cut in a cryostat and mounted on Star Frost adhesive glass slides (Knittelgläser) that were stored at -80°C until use for immunohistochemical analysis.

### 4.3.2 mDC and pDC in inflamed synovium.

Other studies of DC infiltration to the target tissue in inflammatory disease have utilised tissue dissociation and FACS analysis to determine DC subset numbers (622). However, the enzymatic digestion of synovium is associated with increased cell death and altered CD receptor expression, thus compromising the recovery of rare DC subsets and rendering phenotype investigation difficult. In addition this method does not allow examination of tissue architecture and the distribution of DC subsets therein. Accordingly, we first chose to examine the expression and distribution of CD1c<sup>+</sup> mDC and CD304<sup>+</sup>/CD123<sup>+</sup> pDC in synovial tissue taken at biopsy from RA, PsA and OA patients by immunohistochemical methods.

**Table 4.1.** Clinical features of RA, PsA and OA patients included in the study\*

Diagnosis	Characteristic	Median (range)
RA n=20	Age (years) (Median, range)	53 (26-73)
	Male:Female	9:11
	Disease Duration (months)	46 (4-412)
	ESR (mm/hour)	50.5 (4-112)
	CRP (mg/liter)	22.5 (<3-122)
	RF (+/-)	16 (+), 4 (-)
PsA n=9	Age (years) (Median, range)	49 (34-71)
	Male:Female	5:3
	Disease Duration (months)	198 (12-456)
	ESR (mm/hour)	44.5 (7-50)
	CRP (mg/liter)	27.5 (7-51)
	RF (+/-)	0 (+), 9 (-)
OA n=10	Age (years) (Median, range)	69 (54-81)
	Male:Female	1:7 (NA=2)
	Disease Duration (months)	39 (6-240) (NA=2)
	ESR (mm/hour)	18.5 (14-63) (NA=2)
	CRP (mg/liter)	7 (2-60) (NA=2)
	RF (+/-)	0 (+), 8 (-) (NA=2)

\*RA, rheumatoid arthritis; PsA, psoriatic arthritis; OA, osteoarthritis; ESR, erythrocyte sedimentation rate; CRP, C reactive protein; RF, rheumatoid factor; NA, not available  
Median and range is given for each characteristic.

#### **4.3.2.1 RA mDC and pDC synovial tissue distribution**

CD1c<sup>+</sup> mDC and CD304<sup>+</sup>/CD123<sup>+</sup> pDC were dispersed throughout the synovial sublining, but not the superficial lining layer, in RA (n=5) (Fig. 4.1a). CD1c<sup>+</sup> mDC could be identified in close association with other lymphocytes and, to a lesser extent, in peri-vascular regions. CD304<sup>+</sup>/CD123<sup>+</sup> pDC were also identified in close association with other lymphocytes and in peri-vascular regions.

#### **4.3.2.2 PsA mDC and pDC synovial tissue distribution**

CD1c<sup>+</sup> mDC and CD304<sup>+</sup>/CD123<sup>+</sup> pDC were dispersed throughout the synovial sublining, but not the superficial lining layer, in PsA (n=5) (Fig. 4.1b). CD1c<sup>+</sup> mDC could also be identified in peri-vascular regions and in close association with cellular aggregates. CD304<sup>+</sup>/CD123<sup>+</sup> pDC were also identified in close association with other lymphocytes and in peri-vascular regions.

Psoriasis is a significant defining extra-articular characteristic of PsA and pDC have previously been identified infiltrating inflamed skin lesions in studies by other groups (437, 622). Accordingly, we obtained SM matched inflamed skin biopsies from one patient in order to investigate whether both sites could present targets of pDC migration in PsA. For this analysis mDC were identified by CD1c staining, while the pDC specific CD303 was used to identify pDC infiltration. CD1c<sup>+</sup>mDC were identified in the dermal and epidermal layer of skin (Fig. 4.2a) and in the sub-lining layer of matched SM (Fig. 4.2c). CD303<sup>+</sup>pDC were identified in the dermal, but not the epidermal, layer of skin (Fig. 4.2b) and in the sub-lining layer of matched SM (Fig. 4.2d). This suggests that in the case of PsA, inflamed skin also constitutes a target for DC migration. However, analysis of additional matched biopsies would be required in order to undertake informative quantitation of the number of mDC and pDC infiltrating either site.

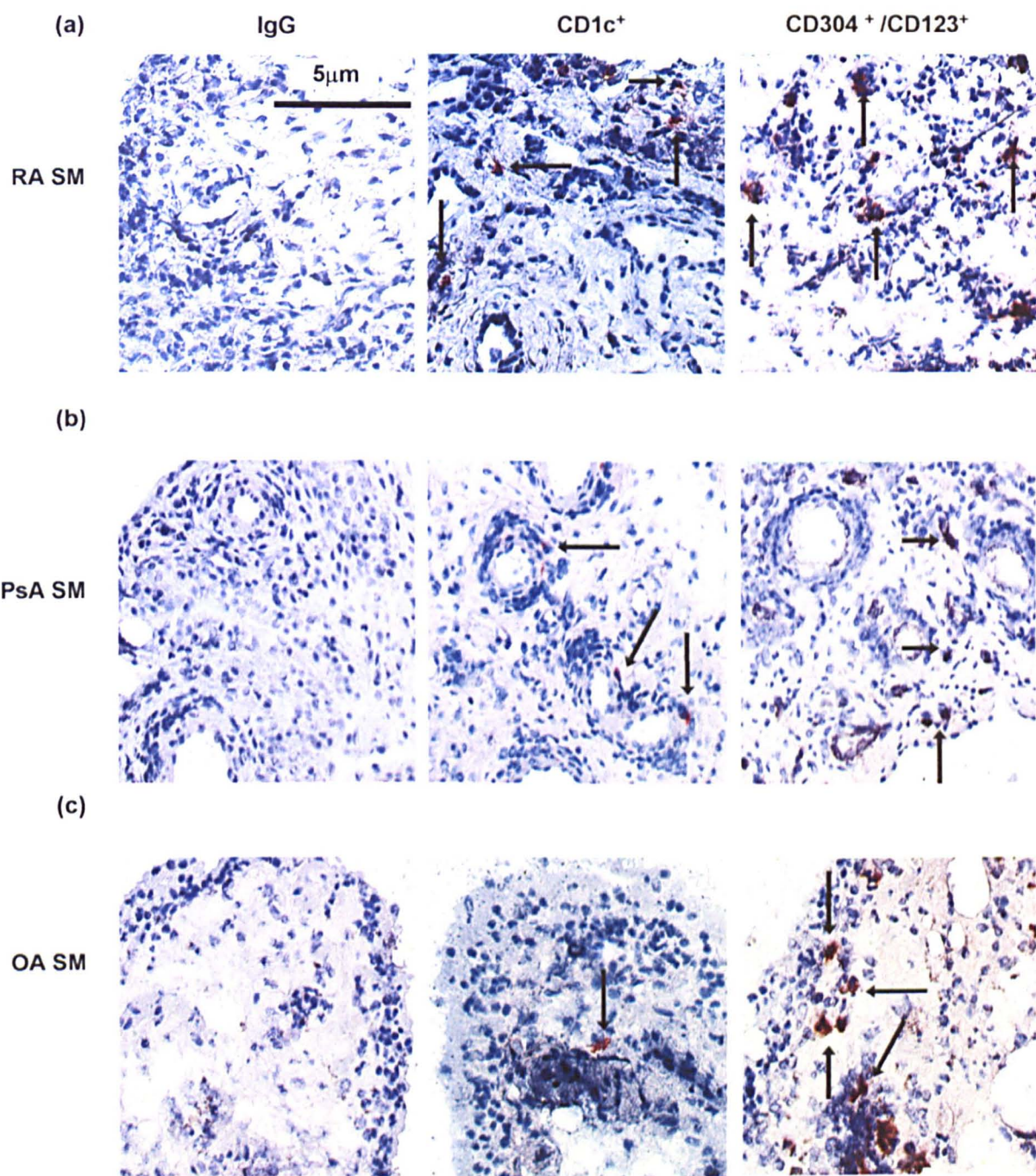
#### **4.3.2.3 OA mDC and pDC synovial tissue distribution**

CD1c<sup>+</sup> mDC and CD304<sup>+</sup>/CD123<sup>+</sup> pDC were dispersed throughout the synovial sublining, but not the superficial lining layer, in OA (n=5) (Fig. 4.1c). CD1c<sup>+</sup> mDC could be observed in close association to other lymphocytes. CD304<sup>+</sup>/CD123<sup>+</sup> pDC were also identified in close association with other lymphocytes. Neither subset was observed in peri-vascular regions, possibly because OA SM did not appear as vascularised as its RA and PsA counterparts.

**Figure 4.1 CD1c<sup>+</sup> mDC and CD304<sup>+</sup>/CD123<sup>+</sup> pDC are present in RA, PsA and OA SM.**

DC subset infiltration to the synovium was investigated by immunohistochemical methods. CD1c<sup>+</sup> mDC were visualized using AEC substrate (stained red). For pDC detection, double-staining was used with CD304<sup>+</sup> cells visualized using DAB substrate (brown) and CD123<sup>+</sup> cells visualized using AEC substrate (red). pDC were identified as CD304<sup>+</sup>/CD123<sup>+</sup> double positive cells, appearing black using this technique. mDC and pDC were identified in the inflammatory cell infiltrate to the sub-lining layer and around vessels of synovial membrane of serial sections taken from RA (n=5) (a), PsA (n=5) (b) and OA (n=5) (c). Examples of positive cells are indicated by arrows. One example of each disease type is shown with isotype control.

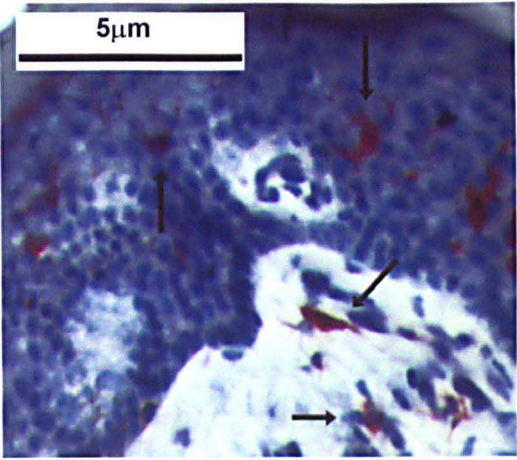




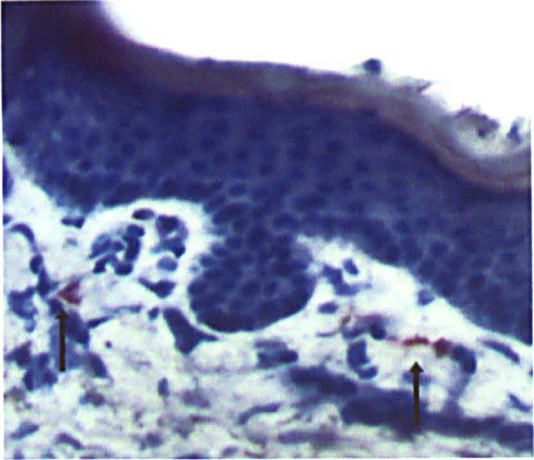
**Figure 4.2 Inflamed psoriatic skin lesions contain CD1c<sup>+</sup> mDC and CD303<sup>+</sup> pDC.**

Single stain CD1c<sup>+</sup>mDC (a) (red) were identified in the dermal and epidermal layer and CD303<sup>+</sup>pDC (b) (red) were identified in the dermal layer of an inflamed psoriatic skin biopsy taken from one patient with active psoriatic arthritis. Single stain CD1c<sup>+</sup>mDC (c) (red) and CD303<sup>+</sup>pDC (d) (red) were also identified in the inflammatory cell infiltrate to the patient matched SM. Examples of positive cells are indicated by arrows. An isotype control is also shown (e).

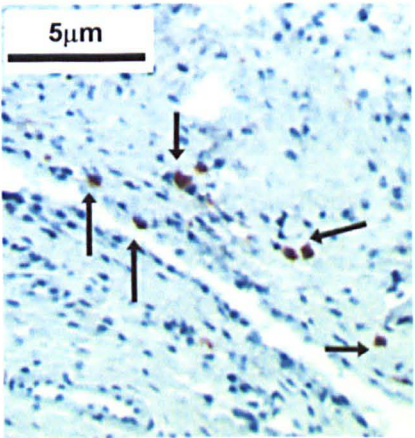
(a)



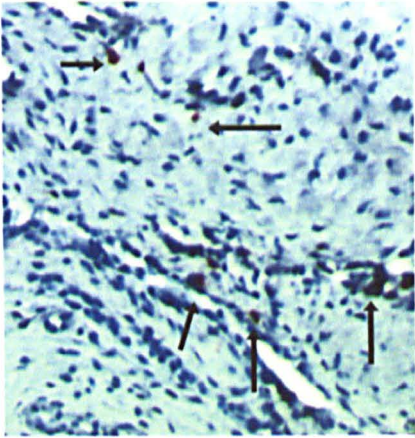
(b)



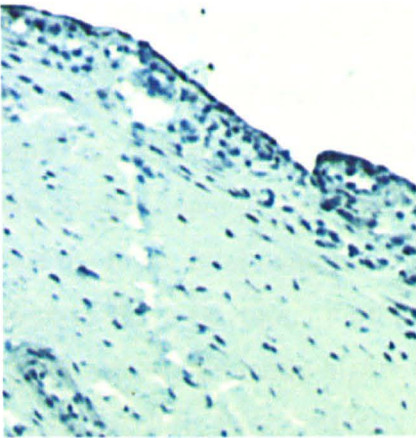
(c)



(d)



(e)



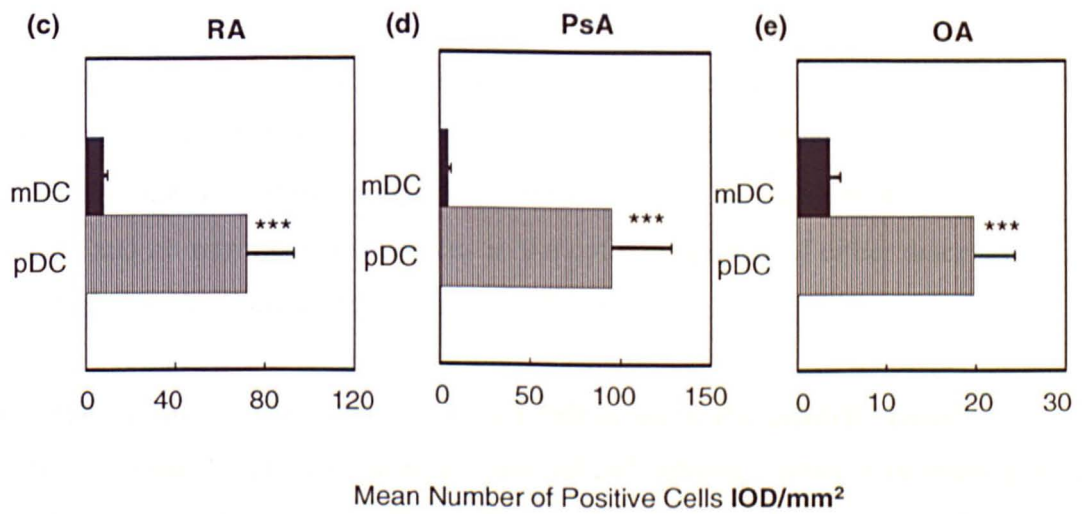
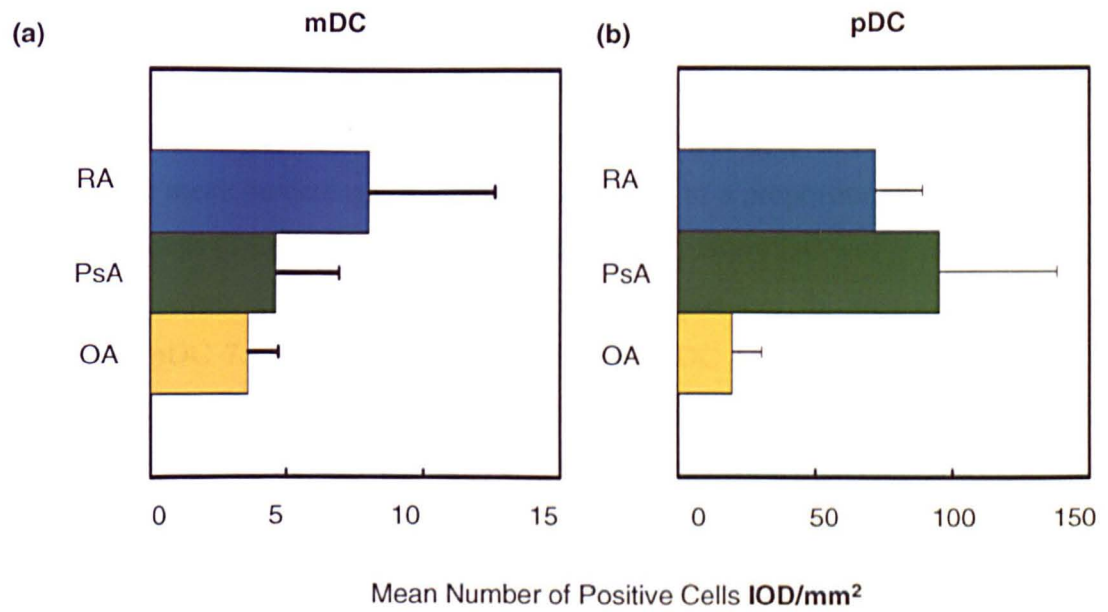
### 4.3.3 Digital image enumeration of pDC and mDC in SM

Digital image analysis was used to quantify the number of pDC and mDC in SM from RA, PsA and OA SM. Digital imaging allows computer-assisted analysis whereby 6 consecutive high-power fields are taken from each of 3 random regions, resulting in 18 high-power fields per section thus providing a representative measurement of the entire tissue section. In order to allow comparative digital image analysis, tissue must be stained in single session. To enable single session processing of a large number of sections, further immunohistochemical identification of pDC in SM was undertaken using single CD304 staining. Although CD303 is a more specific pDC antibody than CD304, staining (as confirmed by use of tonsil sections as positive controls) was inconsistent for light microscopic immunohistochemistry. Technical limitations were, therefore, placed on the volume of tissue sections that could be stained at one time, a problem also experienced by other groups (125, 595). However, whereas others have instead opted to analyse pDC using CD123, which is expressed by numerous cell types (as discussed in Chapter 3), we chose the more specific marker CD304. Although CD304 is also expressed at very low levels on endothelial cells, we observed that these only appeared with the substrate DAB when additive double-staining methods, as utilised for the initial analyses of pDC infiltration to the synovium, were used. Furthermore, CD304<sup>+</sup>/CD123<sup>+</sup> leukocytes, not belonging to either DC subset, were identified during double-staining thus highlighting the importance of using the more specific CD304 antibodies, and not CD123, to accurately identify the respective DC subsets and exclude non-DC leukocytes from digital image analysis.

Digital data image analysis of the number of mDC and pDC infiltrating RA (n=20), PsA (n=9) and OA (n=10) SM revealed that although RA and PsA SM contained more pDC and mDC than OA SM, there were no significant differences between disease groups (Fig. 4.3a and Fig. 4.3b). It is likely that the lower total numbers in OA SM could be explained by a lower total cellular infiltration to the synovium. Furthermore, the number of pDC detected within each diagnostic group was significantly higher compared to the number of mDC in comparable tissue areas (RA,  $P<0.001$ ; PsA,  $P=0.0011$ ; OA,  $P=0.0052$ ) (Fig. 4.3c, Fig. 4.3d and Fig. 4.3e). These findings are consistent with a common pattern of DC infiltration across the three diseases.



**Figure 4.3 RA, PsA and OA SM contain comparable numbers of pDC and mDC between diagnostic groups, but significantly more pDC than mDC within each group.** The number of mDC and pDC infiltrating RA (n=20), PsA (n=8) and OA (n=10) SM was calculated by CD1c (mDC) and CD304 (pDC) staining and computer assisted digital image analysis. No significant differences were observed between the numbers of both mDC (a) or pDC (b) present in RA, PsA and OA SM. In RA alone (c), PsA alone (d) and OA alone (e) the number of pDC was significantly higher than mDC (all \*\*\*<0.001). Results are shown as mean numbers of positive cells integrated optical density (IOD)/mm<sup>2</sup>±SEM. Statistical significance was determined by Mann-Whitney U test (\*\*\*P<0.001).



#### **4.3.4 Inflamed synovium contains more immature than mature mDC and pDC.**

It has been hypothesised that maturing DC may present peptides from dying self-tissue and environmental proteins as well as from pathogens, when encountered in a highly inflamed environment, promoting chronic inflammatory and autoimmune events. The maturation status of mDC and pDC in RA, PsA and OA SM was assessed using CD1c-FITC and CD303-FITC (which works well for immunofluorescence) in combination with the DC maturation markers CD83 (Fig. 4.4a and Fig. 4.4c and Fig. 4.4e) and DC-LAMP (Fig. 4.4b and Fig. 4.4d). Mature mDC and pDC were identified in RA (n=12), PsA (n=7) and OA (n=5) SM with no significant difference in number per area observed between groups. However, the mean percentage of mature CD83<sup>+</sup> DC, as a proportion of all DC, was low in all patient groups (Table 4.2). The low percentage of mature DC was confirmed using DC-LAMP, with even fewer mDC and pDC staining positive for this marker (RA mDC 1.1±1.6 and pDC 7.1±4.3, PsA mDC 2.7±2.2 and pDC 16.1±4.5, OA mDC 5.9±4.8 and pDC 6.2±3.1). These results indicate that over 76% of all mDC and 79% of all pDC in membrane retain an immature phenotype. Few disease differences emerged aside from the % of CD83<sup>+</sup> mDC in RA and PsA compared to OA SM. Of interest, within RA SM only the percentage of CD83<sup>+</sup> pDC was significantly higher in comparison to CD83<sup>+</sup>mDC (p=0.0418).

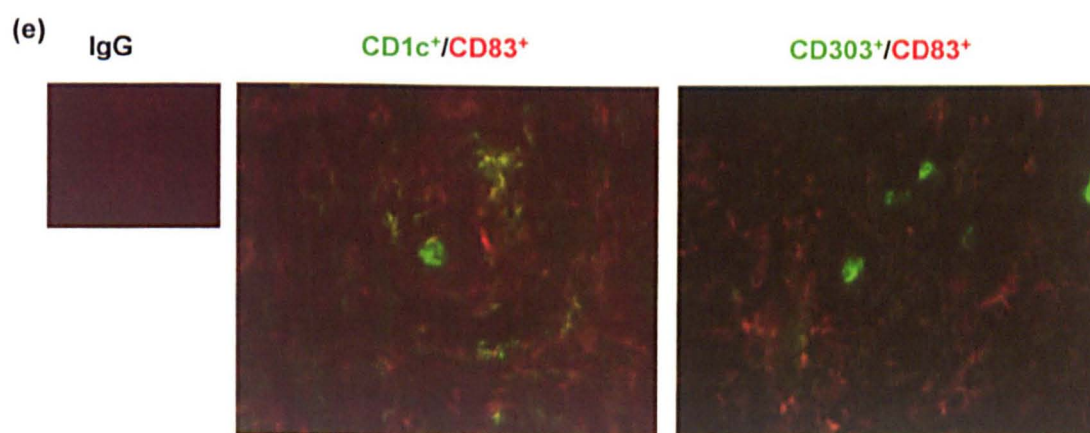
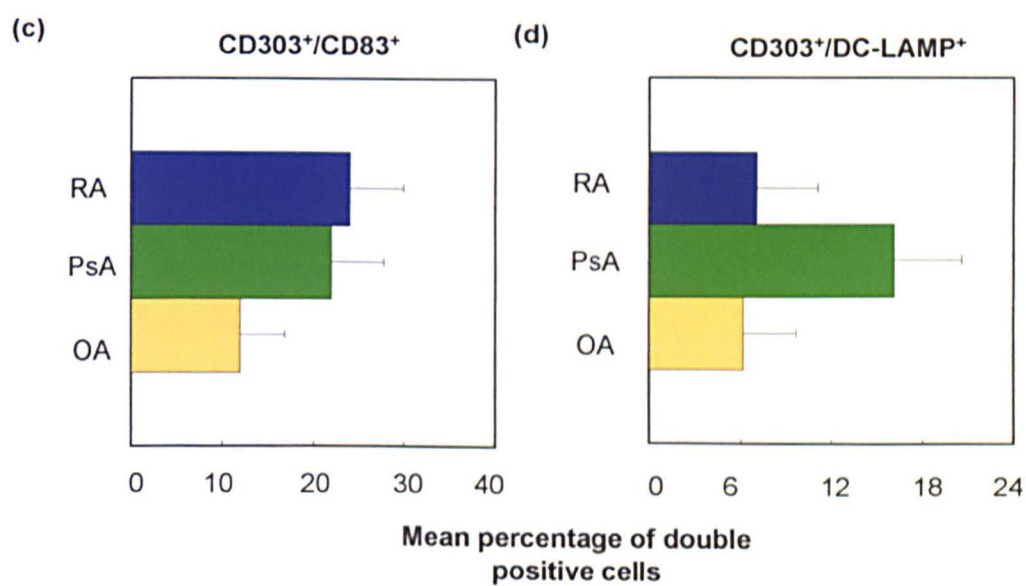
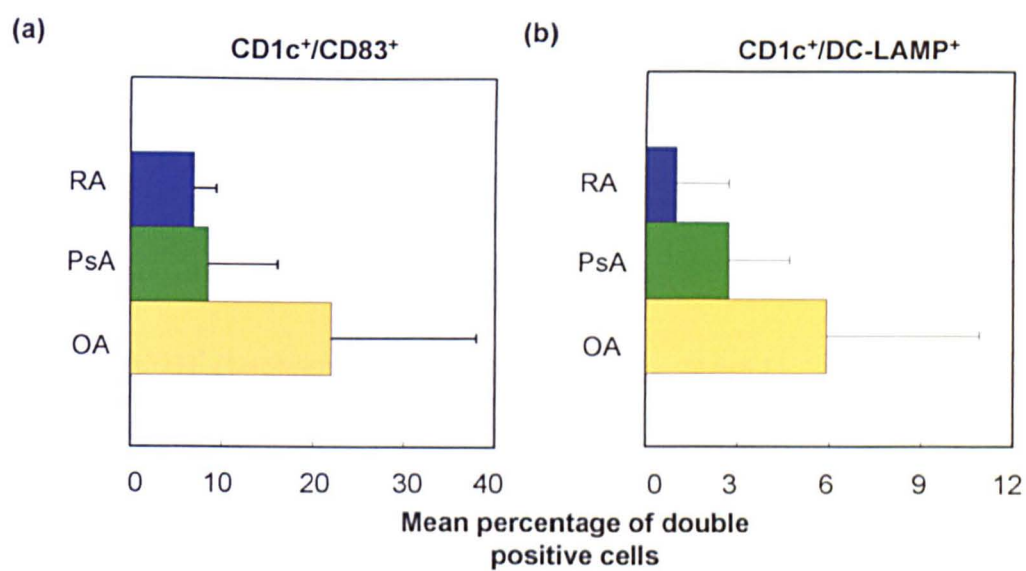
The results of the SM analysis of DC subsets described thus far identified no significant differences between RA, PsA and OA, suggesting that we were observing an inflammatory phenomenon rather than a disease specific observation. This observation is consistent with other studies that identified no significant differences in the inflammatory infiltrate in RA and PsA, and no significant differences in the cytokine profile of either disease (127, 624). Thus the following analyses focus on RA SM only.

#### **4.3.5 mDC and pDC are co-localized to T cell areas in RA synovial tissue.**

We next defined the precise tissue location of DC subsets, particularly relating to T cell subsets given the ability of the former to present antigen and direct the differentiation of the latter. To this end, double immunohistochemistry was performed using antibodies against CD3 and CD8. CD4 was not localized since both CD1c<sup>+</sup> and CD304<sup>+</sup> DC also express the CD4 antigen (397). Both mDC and pDC were identified in close proximity to clusters of CD3 (Fig. 4.5b and Fig. 4.5c respectively) and CD8 (Fig. 4.5d and Fig. 4.5e respectively) positive cells in RA synovial tissue.

**Figure 4.4 RA, PsA and OA SM contain more immature than mature mDC and pDC.** The percentage of CD1c<sup>+</sup> mDC and CD303<sup>+</sup> pDC coexpressing the DC maturation markers CD83 (a and c) or DC-LAMP (b and d) in RA (n=12), PsA (n=7) and OA (n=5) SM was low in all patient groups. A representative double immunofluorescence staining of RA SM from one patient is shown. CD1c<sup>+</sup> mDC (f) and CD303<sup>+</sup> pDC (g) are green, CD83<sup>+</sup> cells are red and double positive cells appear yellow. The isotype control is shown (e). Original magnification x400.



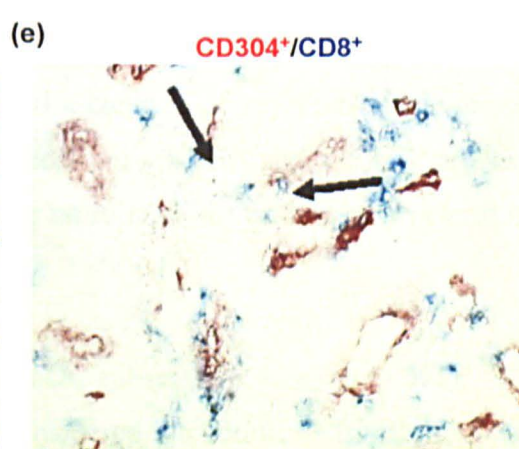
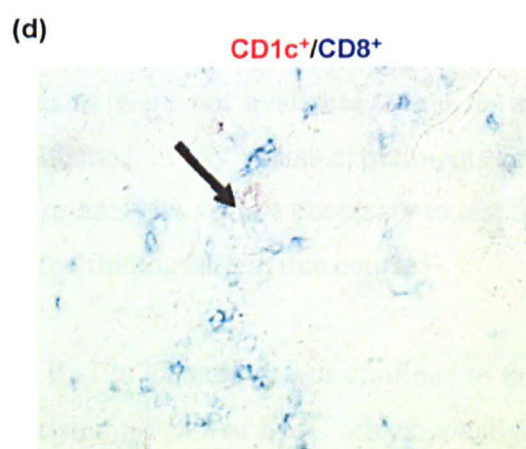
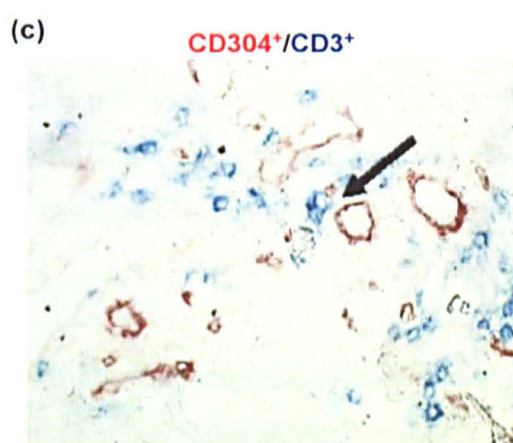
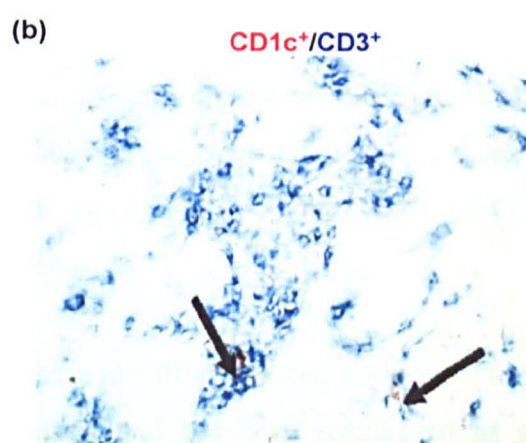
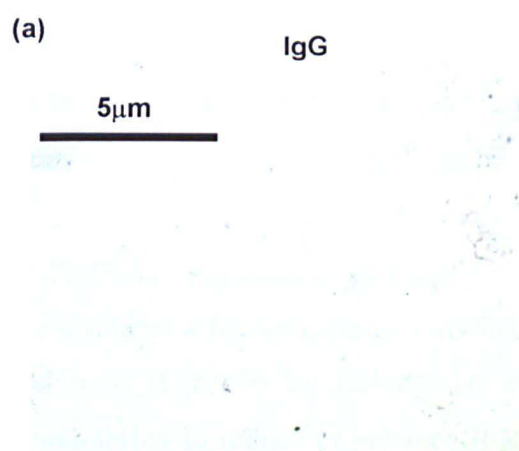


**Table 4.2** Coexpression of the DC maturation marker CD83 by CD1c<sup>+</sup> mDC and CD303<sup>+</sup> pDC in ST from patients with RA, OA and PsA

	RA	PsA	OA
CD1c <sup>+</sup> CD83 <sup>+</sup> (%±SEM)	4.7±2.9	8.9±7.2	23.9±12.1
CD303 <sup>+</sup> CD83 <sup>+</sup> (%±SEM)	23.1±5.9	22.6±6.1	12.7±3.5
P values CD1c vs CD303	0.0418	0.1908	0.2649

**Figure 4.5 mDC and pDC are localized to CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte aggregates in RA SM.**

DC subset localization to lymphocyte aggregates in RA SM was investigated by immunohistochemical methods. CD1c<sup>+</sup> mDC and CD304<sup>+</sup> pDC (red) were visualized using AEC substrate (stained red). For T cell detection, double-staining was used with CD3<sup>+</sup> and CD8<sup>+</sup> lymphocytes visualized using alkaline phosphatase substrate III kit (blue). An isotype control is shown (a). Both CD1c<sup>+</sup> mDC and CD304<sup>+</sup> pDC were identified in close proximity to CD3<sup>+</sup> (b and c) and CD8<sup>+</sup> (d and e) lymphocytes and as CD304<sup>+</sup>/CD123<sup>+</sup> double positive cells, appearing black using this technique. A representative example of RA SM from one patient is shown.



#### 4.3.6 Differential expression of cytokines by mDC and pDC in RA synovial tissue.

The propensity of DC subsets to direct the immune system towards either tolerance or immunity can largely be assessed by their specific cytokine profile. We therefore utilised double immunofluorescent staining techniques to analyse the expression of a number of cytokines of interest by CD11c-FITC<sup>+</sup> mDC and CD303-FITC<sup>+</sup> pDC.

##### 4.3.6.1 *Th1/Th17 regulatory cytokines*

RA is considered a predominantly Th1/Th17 skewed disorder. We therefore analysed the expression of IL-12p70 by DC subsets as its presence in RA SM, coupled with the functional ability to induce or enhance IFN- $\gamma$  production and magnify the Th1 phenotype of disease, has been established by a number of studies (269, 625). We also chose to analyse IL-23p19, a cytokine of particular current interest as it is critical to the local expansion of Th17 T cells, and has been proposed as an essential promoter of end stage joint autoimmune inflammation (329, 626). The p40 subunit of the IL-23 heterodimer (p19/p40) was not measured as this is shared with IL-12 (627). Critically, it is known that DC are capable of producing both of these cytokines given the local environmental conditions.

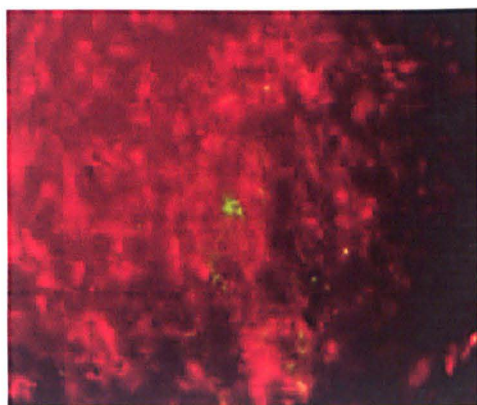
DC subset identification and co-localisation with cytokine expression proved technically challenging and has been recognised as such in many laboratories worldwide in the rheumatology field and beyond. In our experiments, high levels of background staining for IL-12p70, and for type I interferons in particular were observed. New experiments have recently been commenced to address these issues however the reagents and technical refinements were not available to me during the conduct of my thesis experimentation. Data obtained in my initial experiments are contained herein and are reported as such. Future re-analyses will be necessary to test their reproducibility and thereby determine how robust the findings are in due course.

In DC, IL-12p70 staining was confined to the mDC subset (Fig. 4.6a) ( $p < 0.001$ ). This was unsurprising given that mDC are classically considered the dominant IL-12 producing DC subset, although pDC derived IL-12 has been shown *in vitro* following CD40L/CpG ODN motif stimulation (402, 499). Unlike IL-12p70, IL-23p19 was not highly expressed in synovium. However, IL-23p19 expression was significantly higher in mDC than in pDC

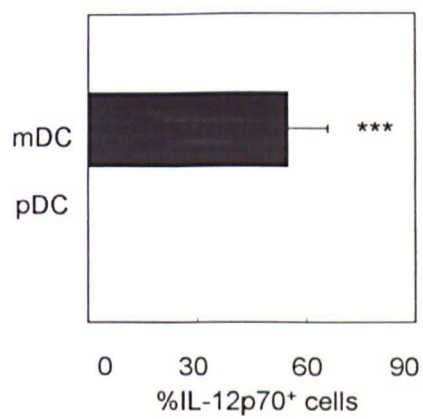
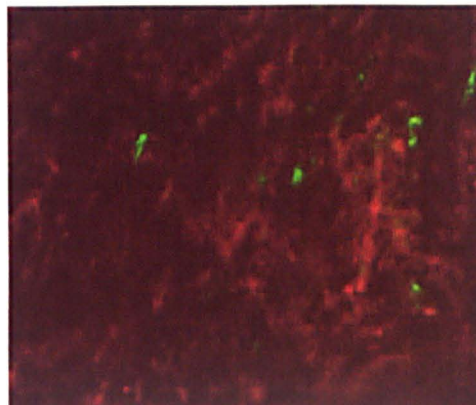
**Figure 4.6 IL-12p70 and IL-23p19 expression by mDC and pDC in SM.**

The expression of IL-12p70 (a) and IL-23p19 (b) by mDC and pDC in serial sections from RA (n=5) was determined by immunofluorescent staining. CD1c<sup>+</sup> mDC and CD303<sup>+</sup> pDC were visualized by FITC-conjugated staining (green), with IL-12p70 and IL-23p19 visualized by Alexa-594 conjugated staining (red). DAPI staining in blue indicates the nucleus. Using this method, mDC and pDC producing either cytokine and thus double positive, appear yellow. One representative section of each stain is shown. Original magnification x400, inset x1000. Statistical significance was assessed by Mann-Whitney U test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

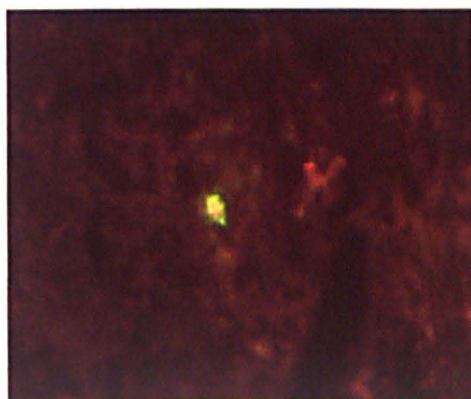
(a) **CD1c<sup>+</sup>/IL-12p70**



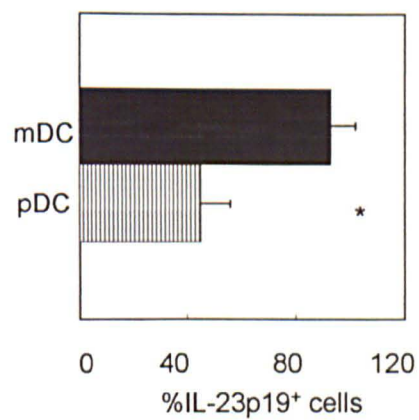
**CD303<sup>+</sup>/IL-12p70**



(b) **CD1c<sup>+</sup>/IL-23p19**



**CD303<sup>+</sup>/IL-23p19**



( $p=0.0273$ ). Interpretation of these data is confounded by the high levels of background which are obvious from the photomicrographs reproduced herein (Fig.4.6). The proportional expression reported represents the observer manual counts performed together by myself and Dr MC Lebre in Amsterdam. The photographs produced were obtained after the manual count and the background predominantly reflects the blanching of fluorescent signal by then and consequent amplification of the red channel leading to the high signal observed. The fluorescent signal was very much clearer at the point of manual counting and for this reason the numbers generated are retained here representing the data obtained. However, future studies will certainly require to reproduce and confirm these observations and quantifications before formal interpretations can be robustly made. Together these data suggest that mDC, not pDC, are the dominant DC subset responsible for directing inflammation in RA SM towards a Th1/Th17 phenotype.

#### ***4.3.6.2 Innate inflammatory cytokines***

Two cytokines of particular interest to this laboratory are IL-15 and IL-18. IL-15 is involved in the proliferation, survival and recruitment of activated T cells as well as proliferation and immunoglobulin synthesis by B cells (628). Similarly, IL-18 is also involved in T cell recruitment, activation and proliferation in addition to being pro-angiogenic and an inhibitor of fibroblast cell death (629). Both IL-15 and IL-18 work synergistically with IL-12 to enhance IFN- $\gamma$  production. We, and others, have previously demonstrated the presence and pathogenic role of both of these cytokines in RA SM (629-631). Given the identification of DC/T cell clusters within synovium, we were particularly interested to investigate the possibility that mDC and pDC could enhance T cell activity by expression of either of these cytokines.

IL-15 (previously identified at high levels in the RA SM) could be identified in non-DC leukocyte aggregates, as well as by DC (Fig. 4.7a). Within DC, proportionally high numbers of mDC and pDC expressed IL-15. Although a greater percentage of pDC than mDC expressed IL-15, this did not attain statistical significance. IL-18 was also observed scattered throughout the synovium in non-DC leukocytes at relatively high levels (Fig. 4.7b). The expression of IL-18 however was significantly higher by pDC as compared to mDC ( $p=0.002$ ), as almost all pDC identified in SM expressed this cytokine.



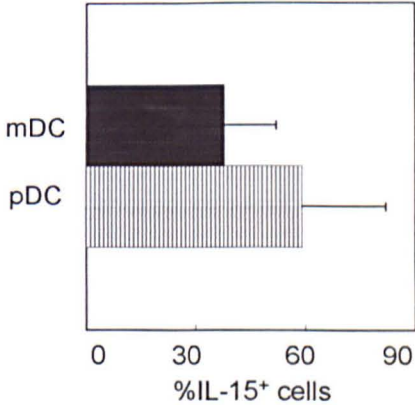
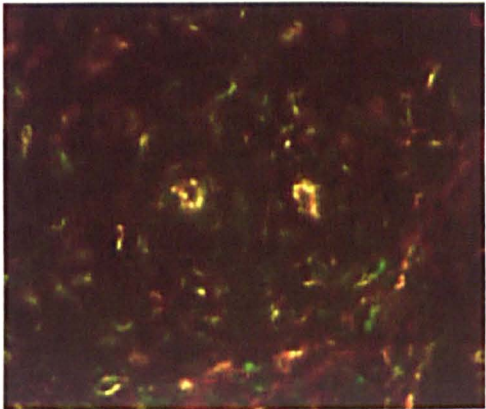
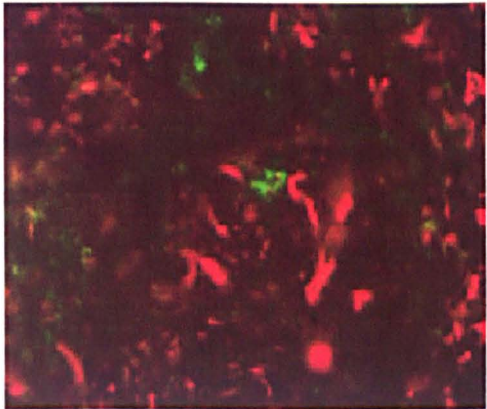
**Figure 4.7 IL-15 and IL-18 expression by mDC and pDC in SM.**

The expression of IL-15 (a) and IL-18 (b) by mDC and pDC in serial sections from RA (n=5) was determined by double immunofluorescent staining. CD1c<sup>+</sup> mDC and CD303<sup>+</sup> pDC were visualized by FITC-conjugated staining (green), with IL-15 and IL18 visualized by Alexa-594 conjugated staining (red). DAPI staining in blue indicates the nucleus. Using this method, mDC and pDC producing either cytokine and thus double positive, appear yellow. One representative section of each stain is shown. Original magnification x400, inset x1000. Statistical significance was assessed by Mann-Whitney U test (\* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ ).

(a)

CD1c<sup>+</sup>/IL-15

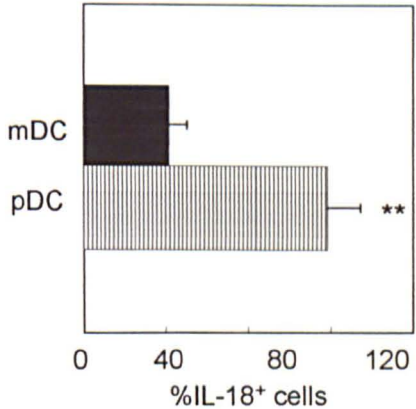
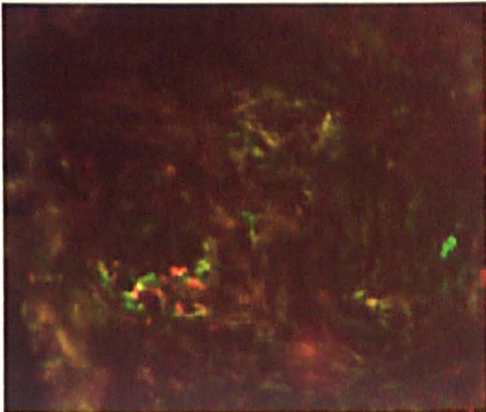
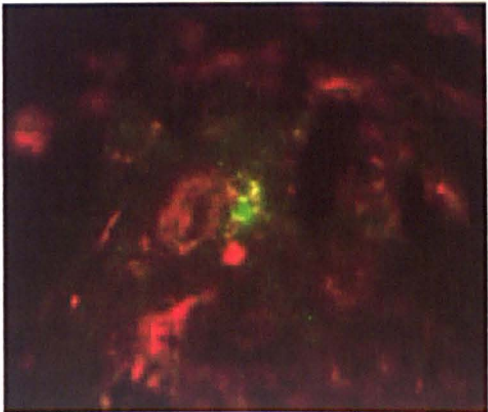
CD303<sup>+</sup>/IL-15



(b)

CD1c<sup>+</sup>/IL-18

CD303<sup>+</sup>/IL-18



#### 4.3.6.3 *Type I IFNs*

The type I IFNs, IFN- $\alpha$  and IFN- $\beta$ , are pleiotropic cytokines that promote isotype switching and have been shown to potently activate autoreactive T cells and contribute to the development of Th1 cells (632, 633). Since IFN- $\alpha$  in particular has been associated with the aetiopathology of other autoimmune diseases including SLE and psoriasis, and pDC are the dominant type I IFN producing cells in the body, we therefore chose to analyse the expression of these cytokines by DC in RA SM.

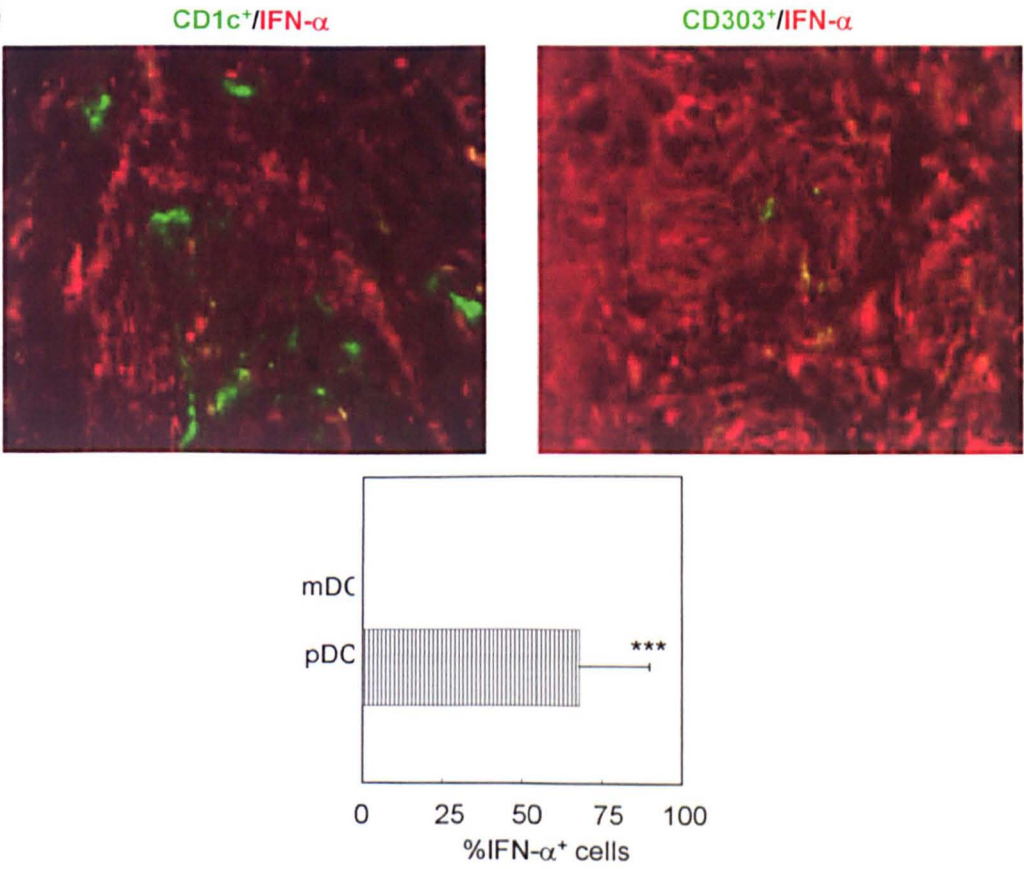
As in the case of IL-12p70, the background staining for the type I IFNs appeared high and as such this analysis needs to be re-visited in order to draw firm conclusions from this data set.

At present, the staining as it was undertaken and scored may offer only an indication of the respective DC type I IFN phenotype within synovium. Analysis suggested the presence of both IFN- $\alpha$  (Fig. 4.8a) and IFN- $\beta$  (Fig. 4.8b) in RA SM. Consistent with classical literature, IFN- $\alpha$  appeared to be expressed by pDC only and not by mDC, with the majority of pDC in SM expressing this cytokine. Conversely, IFN- $\beta$  appeared to be expressed by both DC subsets and although a greater overall percentage of pDC expressed IFN- $\beta$  as compared to mDC, this did not reach statistical significance ( $p=0.0813$ ). Of interest, the type I IFNs have been linked to the inhibition of IL-12 production (634), commensurate with the observation that pDC in synovium do not express IL-12. Subject to the limitations of the photo reproduction these data require to be confirmed and extended prior to robust interpretation – nevertheless the distinct cell subset localisation of IFN- $\alpha$  is consistent with that which we would expect and suggests that properly controlled this approach may offer utility in due course.

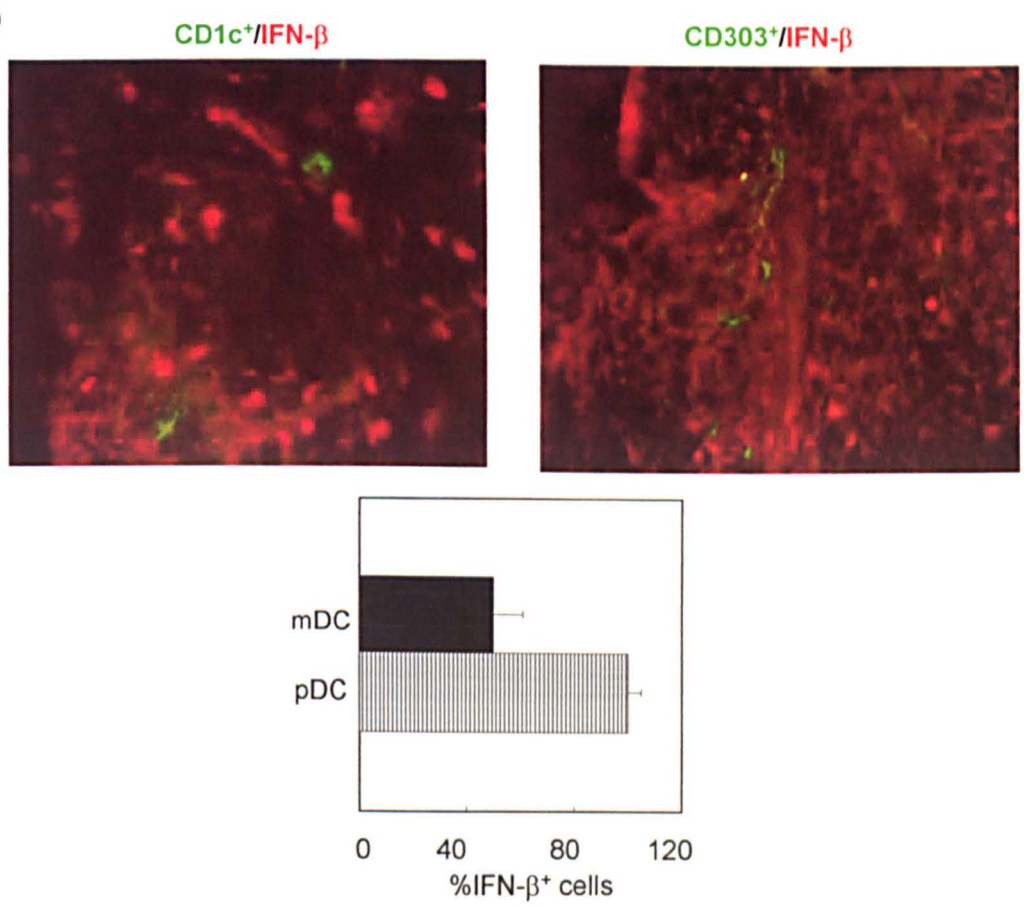
**Figure 4.8 IFN- $\alpha$  and IFN- $\beta$  expression by mDC and pDC in SM.**

The expression of IFN- $\alpha$  (a) and IFN- $\beta$  (b) by mDC and pDC in serial sections from RA (n=5) was determined by double immunofluorescent staining. CD1c<sup>+</sup> mDC and CD303<sup>+</sup> pDC were visualized by FITC-conjugated staining (green), with IFN- $\alpha$  and IFN- $\beta$  visualized by Alexa-594 conjugated staining (red). DAPI staining in blue indicates the nucleus. Using this method, mDC and pDC producing either cytokine and thus double positive, appear yellow. One representative section of each stain is shown. Original magnification x400, inset x1000. Statistical significance was assessed by Mann-Whitney U test (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001).

(a)



(b)



## 4.4 DISCUSSION

Identifying the phenotype, distribution and activation potential of DC subsets in human autoimmune conditions is an important pre-requisite to the description of appropriate tolerance inducing therapies. We now provide detailed information concerning the distribution and phenotype of mDC and pDC within synovial membrane. Moreover such expression was compared between, RA, PsA and OA. Furthermore, and for the first time, using RA SM as an example we show that mDC and pDC possess distinct and unique cytokine profiles.

We identified mDC and pDC infiltration to the synovial sublining in RA, PsA and inflammatory OA SM. The magnitude of this infiltrate did not differ significantly between disease entities. Prior studies by other groups have reported the absence of pDC in OA SM, however these studies analysed OA SM biopsy from just two patients and this limitation may account for the discrepancy (125). It is probable that mDC and pDC are recruited from the circulation to the synovial compartment in response to inflammatory stimuli. mDC and pDC are significantly decreased in the peripheral blood of RA patients and pDC are significantly decreased in the blood of PsA patients (Chapter 3). The possibility that *in situ* differentiation of either subset from a precursor cell lineage driven by the diseased microenvironment, contributed to the synovial accumulation of both mDC and pDC cannot be discounted. This is supported by studies identifying the bone marrow adjacent to the inflamed synovium as an additional disease compartment, particularly as proinflammatory cytokines have been shown not only to promote the release of osteoclast precursors from the bone marrow, but also mediate their differentiation to various cells, including DC (635).

Of particular interest, we make for the first time the remarkable observation that pDC numbers are significantly higher in inflamed synovia than are mDC. The repercussions of this observation are as yet unclear, however it has been hypothesised that IFN- $\alpha$  is a major cytokine culpable for joint damage. Indeed, IFN- $\alpha$  therapy has been linked to the induction of RA and PsA patients with no pre-existing clinical arthritis (545, 619). As pDC are the dominant type I IFN producer in the body and, as we have shown, maintain

this function in the SM, the enrichment of pDC in SM may be indicative of destructive joint disease.

The coexistence of both immature and mature mDC in RA synovium has been reported (274, 534), however there is poor understanding of how DC phenotypes differ between inflammatory arthropathies. In addition, analysis of the maturation status of pDC in SM has not previously been attempted due to technical difficulties (125). Here we show that the majority of the mDC and pDC present in RA, PsA and inflammatory OA SM are immature, as established by low expression of the maturation marker CD83 and confirmed by DC-LAMP. *In vitro* studies of DC differentiation have shown that CD83 up-regulation precedes DC-LAMP up-regulation, indicating that the CD83<sup>+</sup> DC are less mature than their DC-LAMP<sup>+</sup> counterparts (636, 637). This would account for the comparatively reduced DC-LAMP staining, and may indicate a maturation gradient, commensurate with local DC maturation. These findings are consistent with our observation that mDC and pDC maturation is incomplete in inflamed SF (638), and present the intriguing possibility that the SF embodies a source from which membrane DC are derived. Alternatively, the large pool of immature pDC and mDC present in PsA and RA SM might be recruited wholly from the blood in order to mature locally and drive synovial inflammation. It is possible that these DC subsets perpetuate the immune response *in situ* via the continuous presentation of arthritogenic peptides to T cells since they are both localized to T cell lymphocyte aggregates in the RA SM.

To our knowledge, local (RA synovium) cytokine release by mDC and pDC has not yet been investigated. During the acute and chronic inflammatory response, cytokines convey pro- and anti-inflammatory signals between and within cells and as such are crucial in the pathogenesis of RA, as exemplified by the success of cytokine blockade therapies in the clinic.

Analysis of the cytokine profile of both mDC and pDC in RA SM proved technically difficult, with high levels of background staining confounding conclusive analysis. As such, it is acknowledged that this data set requires further examination, in particular as it pertains to the clear identification of individual cells within the synovium. Nevertheless, the analysis as it stands suggests that mDC and pDC within the inflamed RA SM may

display differential cytokine profiles, commensurate with different roles for these cells in the context of synovial inflammation.

IL-23p19 was not highly expressed in the synovium, but where evident was predominantly localised to mDC. IL-12p70 was more abundant in the synovium, although resolution is required to differentiate real staining compared to background staining. Within DC however, IL-12p70 appeared to be confined to mDC. mDC may have a central role in directing the phenotype of T cell mediated inflammation, driving Th1 and IL-17 producing Th17 expansion. IL-17 is a pro-inflammatory cytokine associated with a variety of autoimmune diseases. Its role in arthritic pathology has been shown by murine studies (639, 640). Although the production of IL-23 by DC within SM has been hypothesised by others (641) this is the first study to actually demonstrate expression. This is particularly important given the localisation of mDC within T cell aggregates. However, a number of murine studies have indicated divergent roles for IL-12 and IL-23 in inflammation, the former inhibiting the expansion of Th17 cells, the latter promoting it (642). These studies have postulated that IL-23 induced IFN- $\gamma$  production, enhances IL-12 thus creating self-regulating feedback, the balance of which is reliant on the ratio of IL-12: IL-23. Since more mDC in SM by our analysis were expressing IL-12 it would suggest a Th17 limiting process. However, IL-17 has been measured at elevated levels in the SM and SF of patients with RA suggesting that this negative feedback may not be intact (626, 643, 644). Additional studies will be required to determine if SM derived mDC are dysfunctional in this respect, but pDC and macrophage driven Th17 through IL-23 release can also not be discounted.

We previously demonstrated IL-15 and IL-18 on macrophages, lymphocytes, endothelial cells, and fibroblast-like synoviocytes in RA SM (629-631). We now extend this analysis to include both mDC and pDC. While both DC subsets expressed IL-15 and IL-18 in RA SM, pDC represented the dominant DC source of both. As such, pDC may amplify synovial inflammation since IL-15 and IL-18 are pro-inflammatory and can work in synergy with IL-12 to induce IFN- $\gamma$  production and drive a Th1 phenotype. Of interest, IL-18 has been shown to recruit mDC (645) and pDC (646) to areas of inflammation, in particular under Th1 cytokine conditions, thus DC may provide a self-driven mechanism by which additional DC are recruited to the inflammatory compartment.



Targeting IL-15 has proven therapeutically beneficial in RA, however the mechanisms by which this may occur are not completely defined (341). It has been demonstrated that DC derived IL-2, required to enable T cell contact and proliferation, is tightly co-regulated with the expression of IL-15 (647). Since the majority of mDC and pDC in SM expressed IL-15 and both subsets were localised to T cell aggregates, this cognate interaction may constitute one of the targets of IL-15 therapy by limiting DC driven T cell expansion. The IL-15 driven conversion of monocytes to moDC may also represent a local cytokine-mediated feedback loop driven by SM localised DC and this too, may present an additional target of IL-15 therapy (648).

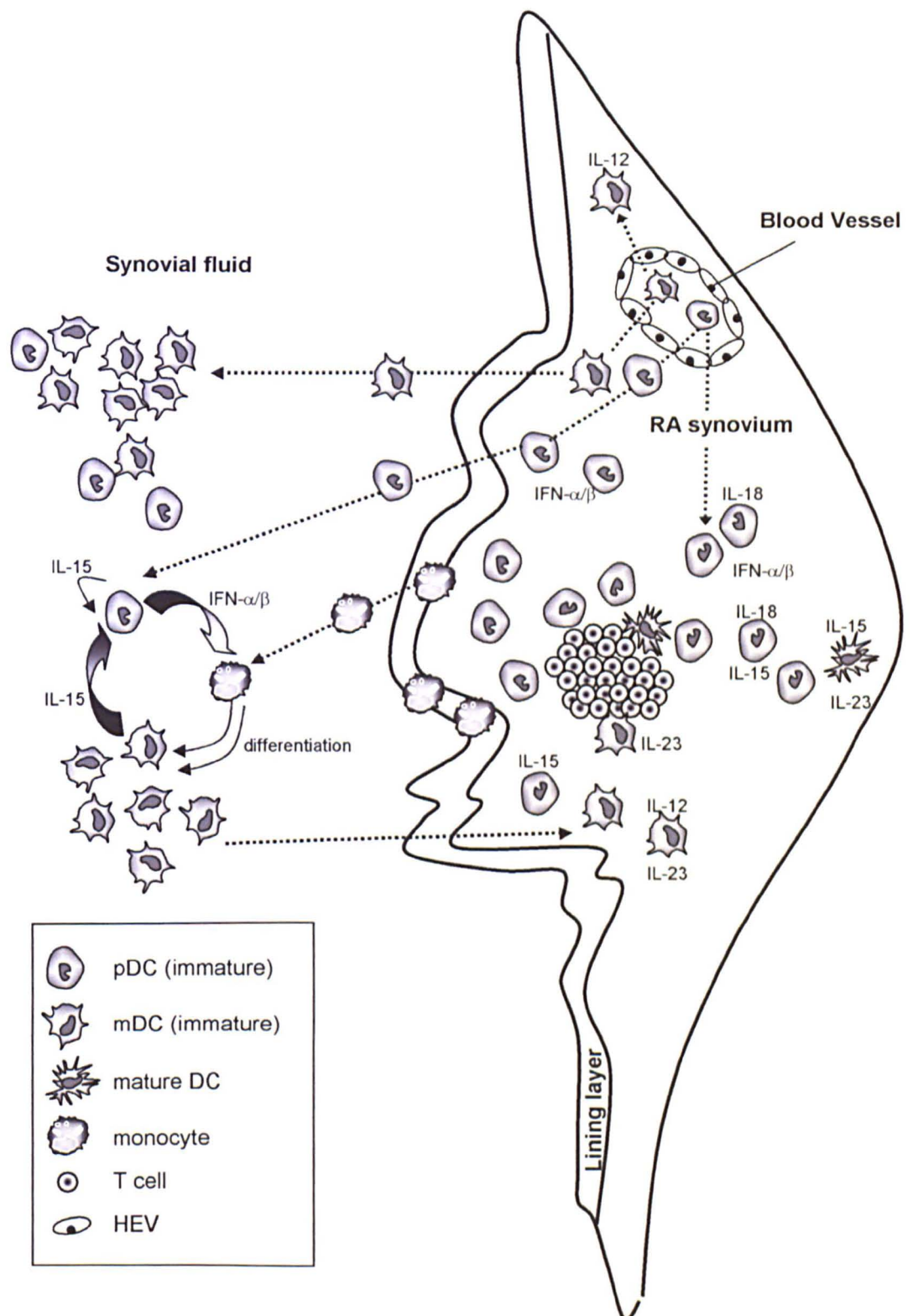
In addition to IL-15, IFN- $\alpha$  has also been ascribed a functional role in the differentiation of monocytes to moDC (498). The analysis reported here suggests that RA synovial pDC are the principal producers of IFN- $\alpha$  and also IFN- $\beta$ , however as in the case of IL-12p70 this data set needs to be re-visited with more refined techniques. Although type I IFN mediated suppression of IL-1 has been shown to limit inflammation (649) and IFN- $\beta$  administration has been shown to be therapeutically beneficial in CIA (650), type I IFNs have also been critically linked to the pathology of various autoimmune diseases including SLE and psoriasis (437, 498). Type I IFNs are pleiotropic cytokines with dual effects, the ultimate function of which is largely determined by the balance of the surrounding cytokine milieu. We propose that within RA SM, given their otherwise pro-inflammatory cytokine profile, DC derived type I IFN may promote inflammation by inducing the development of (auto) antibody-producing plasma cells and activation of autoreactive T cells. In addition, the inflammatory chemokines CXCL10 and CXCL11, chemotactic for pDC, CD4<sup>+</sup> T cells and NK cells, are upregulated in RA SF and SM (349, 651, 652) and the expression of both is inducible by type I IFN (653, 654). This may indicate that, in addition to IL-18, type I IFN may also facilitate pDC infiltration to the SM. Taken together this offers one explanation for the preferential accumulation of pDC to RA SM. Critically, the dominant presence of immature pDC in SM may itself have further pathological ramifications given that IFN- $\alpha$  production is down regulated only following pDC maturation.

Based upon our investigations presented in chapter 3 and chapter 4, we propose a model whereby mDC and pDC migrate from the blood to, and potentially traffic between, the

synovial fluid and synovial membrane (Fig. 4.9). In this model, immature pDC and mDC derived release of pro-inflammatory cytokines may not only contribute to synovial pathology, but also promote further DC recruitment to, and differentiation within, the inflamed synovial compartment. While mature DC of both subsets were detected in SM, the preferential accumulation of immature DC to the SM suggests that either an intrinsic, or extrinsic factor is inhibiting their differentiation. Given that we have already shown that SF purified mDC and pDC are able to mature normally *in vitro*, indicating no intrinsic defect (chapter 3), we propose that as yet unidentified extrinsic factors are responsible for retarding these cells. Of note, the identification of an apparent maturation gradient, progressing from CD83 expression to DC-LAMP expression, indicates that some DC may escape this mechanism and, given via the expression of Th1/Th17 expanding cytokines, lead to sustained lymphocyte activation. The maturation of DC is important to the termination of immune responses as it is coupled by up-regulation of the inhibitory receptor CD200, which allows down-regulation of DC activity. Down-regulation is an active process, an essential requirement for a return to the steady state. It is probable that inhibition of this process could create a 'frustrated' DC phenotype, unable to conclude the immune response. Furthermore, our results are consistent with *in vitro* studies demonstrating that TNF- $\alpha$  limits the maturation of CD34<sup>+</sup> progenitor cultured DC, but also enhances their longevity (655, 656). It is likely that within the proinflammatory synovial environment, with high local concentrations of TNF- $\alpha$ , the DC phenotype becomes skewed, resisting the immunosuppressive effects of IL-10 (458) resulting in reduced chemotactic motility and an abnormally prolonged life span. In future, CD200 expression by DC subsets in synovium will be required to investigate this possibility. The use of anti-CD200R in collagen-induced arthritis has already proved therapeutically beneficial by our laboratory (unpublished communication, McInnes and Gracie) and by others (657), suggesting that disruption of DC function may constitute a therapeutic target. Promisingly, inhibition of the co-stimulation between B7 on APC and CD28 on T cells using CTLA-4 Ig has already proven therapeutically beneficial (381).

**Figure 4.9 Proposed model of mDC and pDC migration from the peripheral circulation into inflamed synovium.**

It is proposed that the migration of pDC and mDC from the peripheral circulation into the inflamed synovial compartment and (in the case of PsA) inflamed skin lesions accounts at least in part for the reduced mDC and pDC numbers in the blood of patients with RA and PsA. Due to yet unknown factors, pDC are preferentially accumulated in synovium compared to mDC, whereas mDC exceed pDC in the SF. The *in situ* release of type I IFNs by pDC may also add to the mDC population by driving differentiation of monocytes (shed from the synovial lining layer into SF) into mDC. This process can be enhanced by autocrine IL-15 production and may explain the large numbers of mDC in SF. Within synovium mDC can direct the immune response through production of IL-12 and IL-15 and, of pivotal importance, expand Th17 T cells through the production of IL-23. pDC production of IL-15 and IL-18 further enhances the synovial inflammation. Since the majority of mDC and pDC in synovium are immature, it is proposed that they display a 'frustrated' phenotype, unable to mature and upregulate inhibitory surface receptors such as CD200, resulting in a state of perpetual activation.



**Chapter 5: Analysis of plasmacytoid dendritic cells and myeloid dendritic cells in RA and PsA synovium 48 hours after TNF blockade and comparative analysis of IL-15 and TNF- $\alpha$  blockade on pDC and mDC cognate interactions with activated T cells *in vitro***

## 5.1 Introduction

The treatment of inflammatory arthritis, in particular RA, has evolved dramatically in the last 15 years. Improvements in biochemistry and molecular biological techniques have allowed the identification of the multiple pro-inflammatory cytokines and enzymes, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-15, IL-17 and matrix metalloproteinases (MMPs), directly culpable for disease pathology. These studies have facilitated the development of novel cytokine targeted therapies, in particular anti-TNF blockade, that have had enormous clinical impact, delaying joint destruction and reducing mortality. Nevertheless, many patients remain refractory to current biological interventions and in those that do respond true remission is rare. A major challenge in therapeutics now is to develop strategies that re-establish immune tolerance such that amelioration of inflammation is accompanied by long-term disease suppression. As the only antigen-presenting cell (APC) recognized to date as capable of provoking autoimmune disease by abolition of self-tolerance, DC therefore represent an attractive therapeutic target.

Pharmacological modulation of DC activation has been demonstrated to prevent disease progression in several T-cell-mediated autoimmune diseases, including RA. Notably, inhibition of DC function by anti-rheumatic drugs including A77 1726, the active metabolite of the potent DMARD leflunomide (556, 557, 658), corticosteroids (659), gold sodium thiomalate (660) and CTLA-4-Ig (571), has been implicated in their therapeutic efficacy. TNF antagonists, including etanercept (a soluble TNF- $\alpha$  type II receptor-IgG<sub>1</sub> fusion protein), infliximab (a chimeric [human and mouse] monoclonal antibody against TNF- $\alpha$ ) and adalimumab (a human anti-TNF monoclonal antibody), are the most efficient therapy for inflammatory arthritis thus far described (661). It is surprising then that their biological effect on DC subsets within the context of human arthritic disease has not been analyzed. Indeed, the broad biological mechanisms of their anti-inflammatory action are not completely understood. Several proposed mechanisms include decreased leukocyte recruitment to the synovium caused by rapid down-regulation of ICAM-1, VCAM-1, CXCL8 and E-selectin (662, 663); decreased local cytokine (664) and MMP (665) production; and inhibition of synovial angiogenesis through reduced VEGF (666).

As we have demonstrated, immature mDC and pDC are preferentially accumulated to the inflamed synovium in active RA and PsA (chapter 4). A number of studies have indicated a role for TNF- $\alpha$  in the differentiation and prolongation of DC in culture (655, 656). It is conceivable that similar mechanisms may operate in arthritis, thus the accumulation to, and apparent static state of development within, the inflamed synovium. Of significance, a biological effect of etanercept on mDC has been demonstrated for psoriasis, the defining extra-articular feature of PsA. It was found that after subcutaneous etanercept administration there was reduced CD11c<sup>+</sup> DC infiltration to, and activity within, psoriatic plaques (569). It is therefore possible that TNF neutralization may exert some of its anti-inflammatory effects directly through mDC. The effect of TNF neutralization on pDC has not yet been assessed. Elucidating the mechanisms of anti-TNF action is critical to advance its optimum use – anti TNF is expensive and not well-tolerated. It may also help to identify possible complementary therapies, such as methotrexate which is already proving efficacious.

One such promising biologic is anti-IL-15. IL-15 is abundant in the inflamed synovium (630) and is not decreased by TNF blockade, indicating that it is not down-stream of the TNF cascade (667). As we have demonstrated in chapter 4, both pDC and mDC in inflamed synovium express relatively high levels of IL-15. IL-15 is critical to DC/T cell contact, tightly co-regulating the expression of IL-2, which is required to enable clonal T cell expansion (647). Because pDC and mDC in the synovium can be co-localized to T cell aggregates, inhibition of cell contact may constitute an important point at which IL-15 blockade breaks self-perpetuating chronic inflammation.

## **5.2 Aims and Objectives**

As described in Chapter 3 and 4, we have identified an accumulation of functionally active, but aberrantly matured pDC and mDC to the inflamed synovial compartment. Previous studies by other groups have demonstrated a critical role for TNF- $\alpha$  in the differentiation of mDC from CD34<sup>+</sup> precursors. Moreover, these studies indicated that TNF- $\alpha$  prolonged the life of DC, but significantly retarded the ability of the cells to mature normally. Given the high concentrations of TNF- $\alpha$  in inflamed SM, and our description of predominantly immature DC in SM, TNF- $\alpha$  may have an aberrant effect on DC in SM. In addition we

identified DC of both subsets in close proximity to T cell aggregates within synovium. IL-15 is required to enable DC/T cell contact, and since the majority of cells from both subsets were expressing IL-15 in SM, inhibiting IL-15 may retard this interaction. It was therefore of particular interest to analyse the effects of TNF- $\alpha$  and IL-15 neutralisation on mDC and pDC.

Specifically, we planned to:

- Analyse the effect of TNF blockade (infliximab) on DC subsets in RA and PsA synovium 48 hours after first infusion by immunohistochemistry on serial synovial biopsy sections.
- Examine the broad biological mechanisms of IL-15 neutralisation in order to ascertain the points in DC/T cell contact at which it may exert its action.
- Comparatively analyse the effect of TNF and IL-15 neutralisation on mDC and pDC in co-culture with T cells, in order to determine if there are any identifiable and specific differences in the cytokine secretion profile after blockade.



## 5.3 Results

### 5.3.1 Infliximab study

#### 5.3.1.1 Patients

Twenty-two patients with active RA (23), defined as a Disease Activity Score (28 joints; - DAS28) of at least 4.8 (at the screening visit), despite maximal methotrexate (either 25mg/week or the maximum tolerable dose) were included in the study. All patients fulfilled the ACR criteria for RA and had failed treatment with at least 2 DMARDs. Stable prednisolone therapy at a dosage of  $\leq 10$ mg/day and nonsteroidal anti-inflammatory drug (NSAID) treatment were allowed. Twelve PsA (572, 573) patients with active skin disease and active joint inflammation were also evaluated in this study. Active skin disease was defined as at least two psoriatic plaques. Active joint disease was defined as at least three tender joints (maximum 28 joint count and included both ankles) and physician's assessment as moderate or worse, despite maximal methotrexate (either 20mg/week or the maximal tolerable dose). In the PsA group stable dose of NSAID was allowed, but prednisolone therapy was not. All patients gave informed consent and the study protocol was approved by the Medical Ethics Committee of the AMC, University of Amsterdam (Patient characteristics, Table 5.1).

The RA and PsA patients were randomized to receive either 3mg/kg infliximab (RA, n=13; PsA, n=6) or placebo (RA, n=9; PsA, n=6) intravenously. All patients were subjected to small bore arthroscopy performed under local anesthesia of the same swollen index joint (knee, wrist or ankle), immediately before initiation of treatment and 48 hours thereafter. At least 6 biopsy samples were obtained using 2 mm grasping forceps (Storz,). Tissues were snap frozen en bloc in Tissue Tek OCT (Miles Elkhart) and stored in liquid nitrogen until sectioning. Sections (5 mm) were cut in a cryostat and mounted on glass slides (Star Frost adhesive slides), which were stored at  $-80^{\circ}\text{C}$  until immunohistochemical analysis was performed. The study was a single centre, double blind, randomised, placebo controlled study.

**Table 5.1.** Clinical features of RA and PsA patients included in the study\*

Characteristic		Infliximab	Placebo
RA n=22	Age (years) (Median, range)	52 (24-81)	55.7 (37-69)
	Male:Female	3:10	3:6
	Disease Duration (months)	256 (16-765)	22.0 (3-168)
	DAS	6.5 (5.6-8.0)	6.1 (4.9-8.0)
	CRP (mg/liter)	12.5 (3-150)	16.3 (3-97)
	RF positive %	77	55
	MTX dose (mg/week)	16.2 (5-30)	16 (5-30)
PsA n=12	Age (years) (Median, range)	53 (35-70)	45 (26-60)
	Male:Female	3:3	3:3
	Duration of joint disease (years)	9 (5-13)	9 (1-22)
	Duration of skin disease (years)	24 (10-41)	17 (2-28) (NA=1)
	DAS	6.8 (5.0-7.9)	5.7 (4.8-8.2)
	CRP (mg/liter)	23 (10-36) (NA=1)	28 (7-65)
	MTX dose (mg/week)	10 (5-20)	10 (5-20)

\*RA, rheumatoid arthritis; PsA, psoriatic arthritis; CRP, C reactive protein; RF, rheumatoid factor; MTX, methotrexate; NA, not available  
Median and range is given for each characteristic.

### **5.3.1.2 Expression of DC in SM pre and post infliximab infusion**

Previous studies by the AMC have demonstrated reduced leukocyte infiltration to the inflamed RA and PsA synovium as early as 48 hours after infliximab infusion (668, 669). We therefore investigated the early effects of TNF- $\alpha$  blockade on DC subsets in synovium by immunohistochemical analysis of serial synovial biopsies taken from patients with RA (n=22) or PsA (n=12) immediately prior to, and 48 hours after, infusion with 3mg/kg infliximab or placebo. To enable single session processing of a large number of sections followed by comparative data image analysis, mDC were identified by CD1c staining and pDC were identified by CD304 staining. The severity of the inflammatory infiltrate in synovial tissue at baseline was comparable between RA infliximab and placebo treatment groups, and between PsA infliximab and placebo treatment groups.

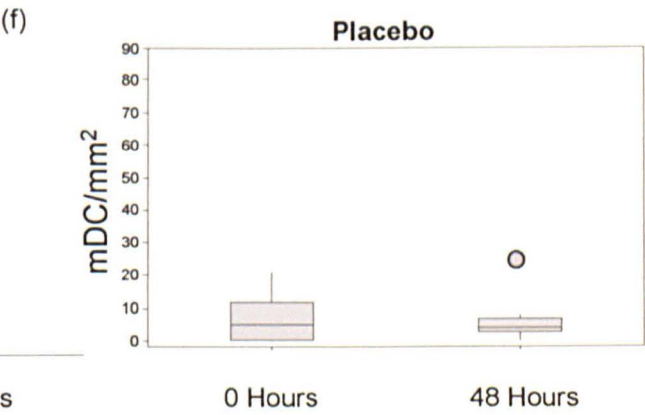
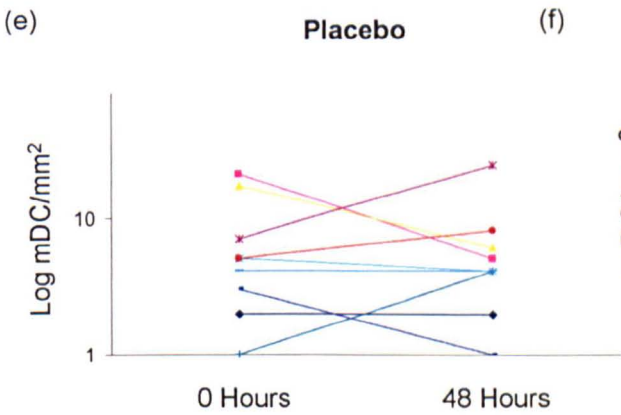
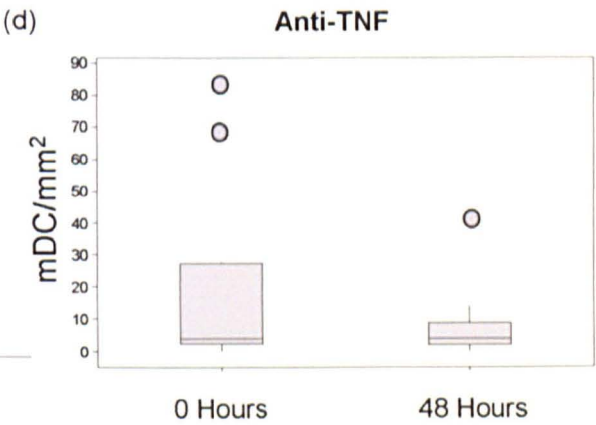
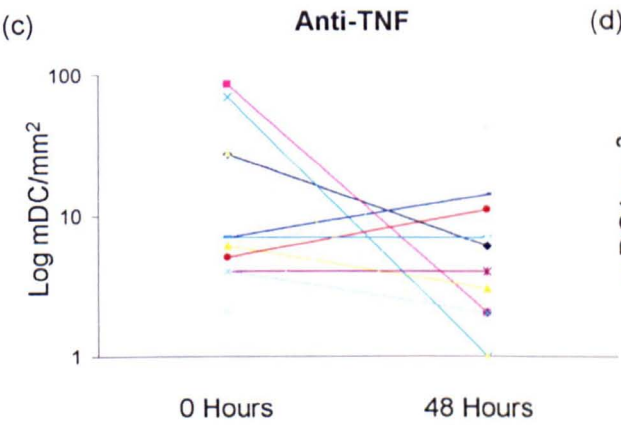
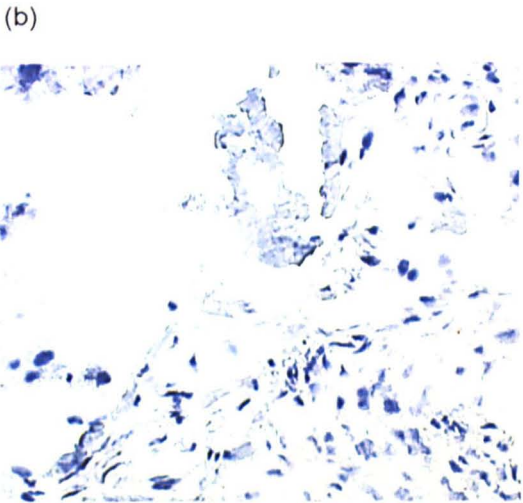
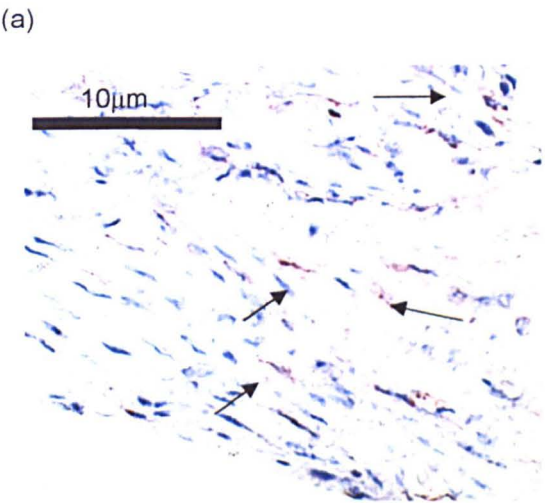
### **5.3.1.3 pDC and mDC in RA SM pre and post infliximab infusion**

The mDC infiltrate in the inflamed synovium was slightly, but not significantly, higher in the infliximab group (n=13) compared to the placebo group (n=9). The difference is illustrative of the inter-individual variability inherent in this heterogeneous disease (134). There was a trend towards reduced mDC number in SM 48 hours after infliximab infusion, as compared to baseline, however this did not attain statistical significance (baseline, median = 13.5 (3-36.5) cells/mm<sup>2</sup>; 48 hours, median = 5 (2-10.50) cells/mm<sup>2</sup>, p=0.272) (Fig. 5.1c and 5.1d). The number of mDC in SM of the placebo group was not reduced 48 hours after infusion compared to baseline (baseline, median = 5.0 (0.5-12) cells/mm<sup>2</sup>; 48 hours, median = 4.0 (3.0-7.0) cells/mm<sup>2</sup>, p=0.8598) (Fig. 5.1e and 5.1f).

As with mDC, when the cohort was examined as a whole, the pDC infiltrate to the inflamed synovium was slightly greater, but not significantly so, in the infliximab group compared to the placebo group at baseline. In contrast to mDC, a trend towards increased pDC in SM 48 hours after infliximab infusion, as compared to baseline was observed (baseline, median = 2.0 (1-5.0) cells/mm<sup>2</sup>; 48 hours, median = 4.0 (1.0-7.5) cells/mm<sup>2</sup>, p=0.8375) (Fig. 5.2c and 5.2d). The number of pDC in SM of the placebo group was not altered between baseline and 48 hours after infusion (baseline, median = 2.0 (0.5-3.0) cells/mm<sup>2</sup>; 48 hours, median = 2.0 (0.5-4.0) cells/mm<sup>2</sup>, p=0.5365) (Fig. 5.2e and 5.2f). We note that in this patient cohort the numbers of pDC in RA SM were lower than observed in previous studies (chapter 4).

**Figure 5.1 CD1c<sup>+</sup>mDC in paired RA synovium before and 48 hours after infliximab or placebo infusion.**

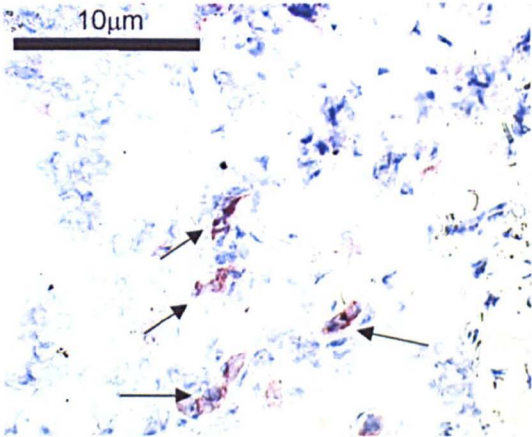
The number of CD1c<sup>+</sup>mDC infiltrating paired serial RA SM biopsies taken before and 48 hours after treatment with infliximab (3mg/kg) (n=13) or placebo (n=9) was detected by immunohistochemical methods. CD1c<sup>+</sup>mDC were visualized using DAB substrate (stained brown) and infiltration enumerated by computer assisted digital image analysis. One example of paired RA SM before infliximab infusion (a), showing CD1c<sup>+</sup>mDC dispersed throughout the synovial sublining layer, and after 48 hours (b), showing a decrease in CD1c<sup>+</sup>mDC, is shown. In the infliximab group the trend was towards an overall decrease in the mDC infiltrate to synovium, although this did not attain statistical significance (c and d). In the placebo group there was no clear trend towards increase or decrease in the mDC infiltrate (e and f). The graphs on the left show the total CD1c<sup>+</sup> cells/mm<sup>2</sup> in each patient. The box and leaf plots on the right depict the median and interquartile range (50% of the data), flanked by whiskers indicating the extent of the entire patient group. Circles indicate outliers as determined using MiniTab.



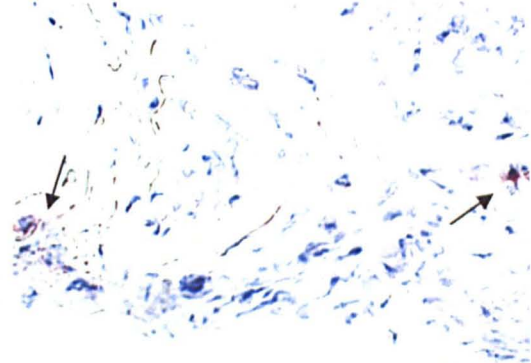
**Figure 5.2 CD304<sup>+</sup>pDC in paired RA synovium before and 48 hours after infliximab or placebo infusion.**

The number of CD304<sup>+</sup>pDC infiltrating paired serial RA SM biopsies taken before and 48 hours after treatment with infliximab (3mg/kg) (n=13) or placebo (n=9) was calculated by immunohistochemical methods. CD304<sup>+</sup>mDC were visualized using DAB substrate (stained brown) and infiltration enumerated by computer assisted digital image analysis. One example of paired RA SM showing CD304<sup>+</sup>pDC localized to the synovial sublining layer before (a) and 48 hours after (b) infliximab infusion is shown. In the infliximab group the overall trend was towards an increase in the pDC infiltrate to synovium, although this did not attain statistical significance (c and d). In the placebo group there was no clear trend towards increase or decrease in the pDC infiltrate (e and f). The graphs on the left show the total CD304<sup>+</sup> cells/mm<sup>2</sup> in each patient. The box and leaf plots on the right depict the median and interquartile range (50% of the data), flanked by whiskers indicating the extent of the entire patient group. Circles indicate outliers as determined using MiniTab.

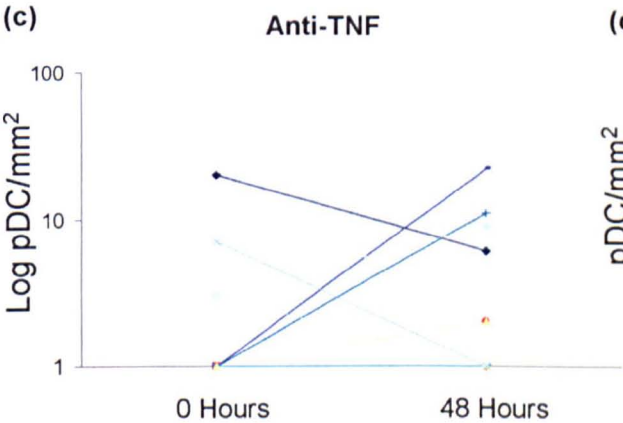
(a)



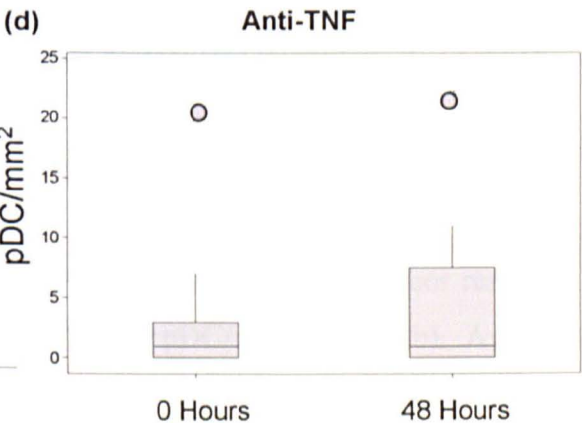
(b)



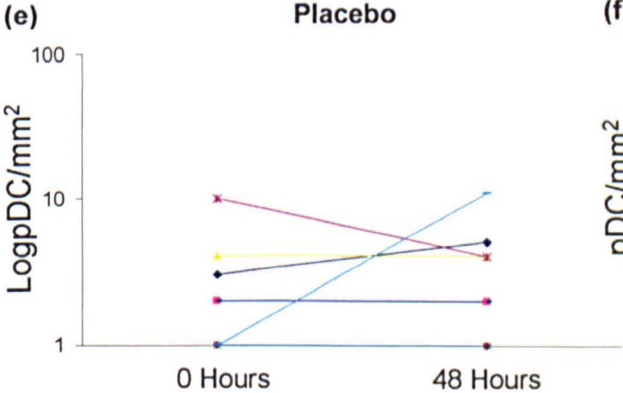
(c)



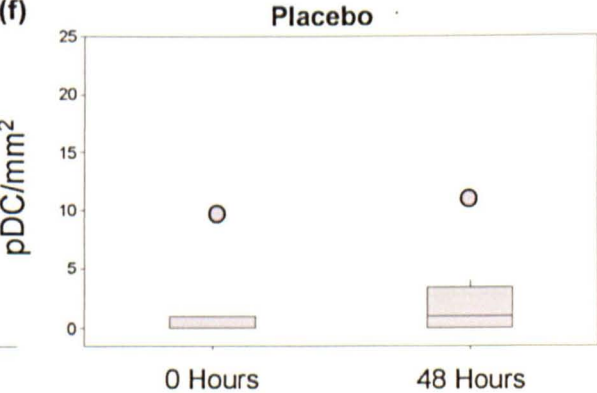
(d)



(e)



(f)



#### **5.3.1.4 pDC and mDC in RA patients responsive to infliximab infusion**

Approximately 50% of patients are refractory to TNF blocking treatment, failing to achieve a 50% improvement in disease activity according to the ACR criteria (ACR50) (368, 670). Therefore we next chose to investigate if responsiveness to infliximab therapy correlated to the mDC and pDC infiltrate in SM 48 hours after infusion. Responsiveness was assessed as good (n=1), moderate (n=11) or poor (n=1) as evaluated at 3 months post initial treatment.

After 48 hours, mDC in the good response patient group were reduced from 69 mDC/mm<sup>2</sup> to 1 mDC/mm<sup>2</sup>; in the moderate response cohort mean mDC were reduced from 12.7 mDC/mm<sup>2</sup>  $\pm$  7.48 mDC/mm<sup>2</sup> to 8.5 mDC/mm<sup>2</sup>  $\pm$  3.58 mDC/mm<sup>2</sup>; and in the poor response patient mDC were reduced from 27 mDC/mm<sup>2</sup> to 6 mDC/mm<sup>2</sup> (Fig. 5.3a). Together the data indicate that mDC are reduced in RA SM following infliximab infusion, irrespective of patient responsiveness to treatment. Since the majority of patients responded moderately to treatment, study on a larger scale would be required to confirm this finding.

After 48 hours, pDC in the good response patient were unchanged from 2.0 pDC/mm<sup>2</sup> to 2.0 pDC/mm<sup>2</sup>; in the moderate response cohort mean pDC were increased from 1.5 pDC/mm<sup>2</sup>  $\pm$  2.11 pDC/mm<sup>2</sup> to 4.4 pDC/mm<sup>2</sup>  $\pm$  6.97 pDC/mm<sup>2</sup>; and in the poor response patient group pDC were reduced from 20 pDC/mm<sup>2</sup> to 6.0 pDC/mm<sup>2</sup> (Fig. 5.3b). Again, a study on a larger scale, including a patient cohort with more varied treatment response, would be required to investigate this result further.

#### **5.3.1.5 pDC and mDC in PsA SM pre and post infliximab infusion**

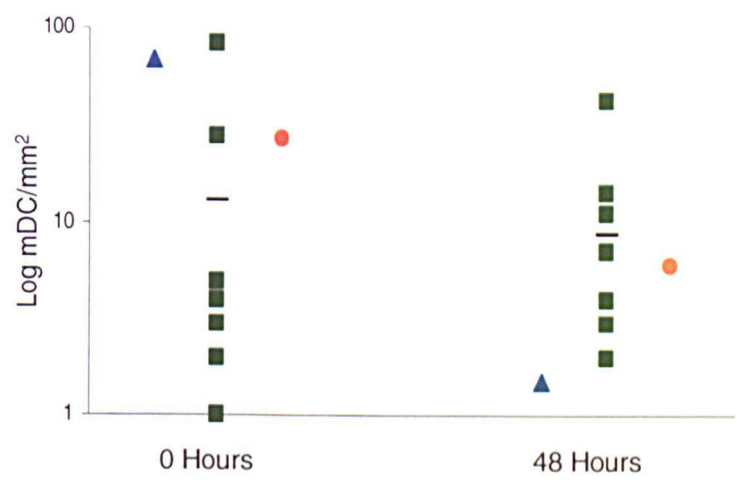
The mDC infiltrate to the inflamed PsA synovium was comparable between the infliximab group (n=6) compared to the placebo group (n=6) at baseline (p = 0.2556). An overall trend towards reduced mDC in SM 48 hours after infliximab infusion, as compared to baseline was observed, however this did not attain statistical significance (baseline, median = 11.9 (1-34) cells/mm<sup>2</sup>; 48 hours, median = 6.0 (1-35) cells/mm<sup>2</sup>, p=0.8983) (Fig. 5.4c and 5.4d). Surprisingly, the number of mDC in SM of the placebo group also indicated an overall trend to reduction 48 hours after infusion compared to baseline (baseline, median = 20 (2-43) cells/mm<sup>2</sup>; 48 hours, median = 6.4 (1-15) cells/mm<sup>2</sup>, p=0.2506) (Fig. 5.4e and 5.4f).



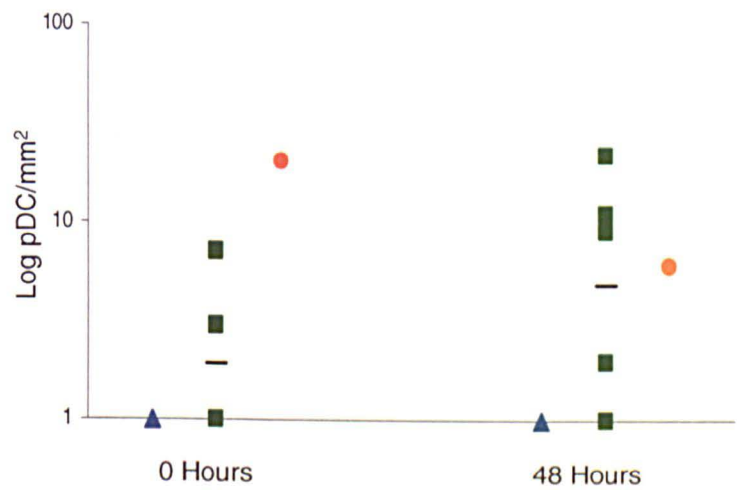
**Figure 5.3 CD1c<sup>+</sup>mDC and CD304<sup>+</sup>pDC in paired RA synovium from good, moderate and non-responders to infliximab, before and 48 hours after infusion.**

The number of CD1c<sup>+</sup>mDC (a) and CD304<sup>+</sup>pDC (b) infiltrating paired serial RA SM biopsies taken before and 48 hours after infliximab infusion was compared between good (triangle, n=1), moderate (square, n=11) and non-responder (circle, n=1) to treatment. Total cells/mm<sup>2</sup> is shown. Line indicates median value.

(a)



(b)

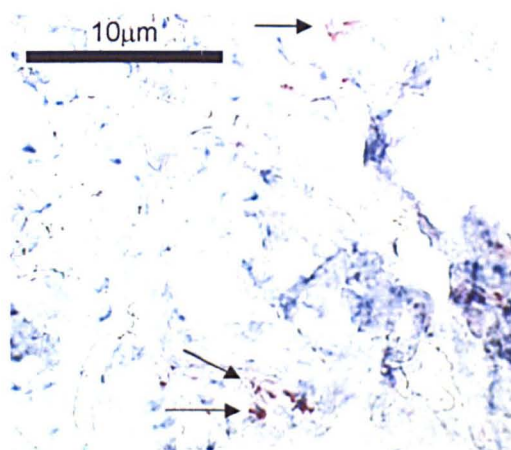


- ▲ Good Response (n=1)
- Moderate Response (n=11)
- No Response (n=1)

**Figure 5.4 CD1c<sup>+</sup>mDC in paired PsA synovium before and 48 hours after infliximab or placebo infusion.**

The number of CD1c<sup>+</sup>mDC infiltrating paired serial PsA SM biopsies taken before and 48 hours after treatment with infliximab (3mg/kg) (n=6) or placebo (n=6) was detected by immunohistochemical methods. CD1c<sup>+</sup>mDC were visualized using DAB substrate (stained brown) and infiltration enumerated by computer assisted digital image analysis. One example of paired PsA SM before infliximab infusion (**a**), showing CD1c<sup>+</sup>mDC in the synovial sublining layer, and after 48 hours (**b**), showing a decrease in CD1c<sup>+</sup>mDC, is shown. In the infliximab group the trend was towards an overall decrease in the mDC infiltrate to synovium, although this did not attain statistical significance (**c** and **d**). In the placebo group the trend was towards a decrease in the mDC infiltrate (**e** and **f**). The graphs on the left show the total CD1c<sup>+</sup> cells/mm<sup>2</sup> in each patient. The box and leaf plots on the right depict the median and interquartile range (50% of the data), flanked by whiskers indicating the extent of the entire patient group. The circle indicates outliers as determined using MiniTab.

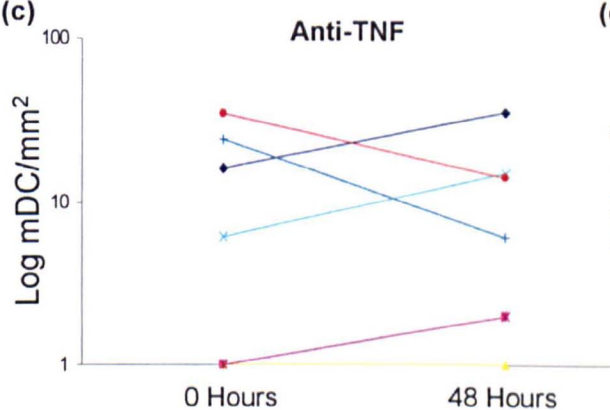
(a)



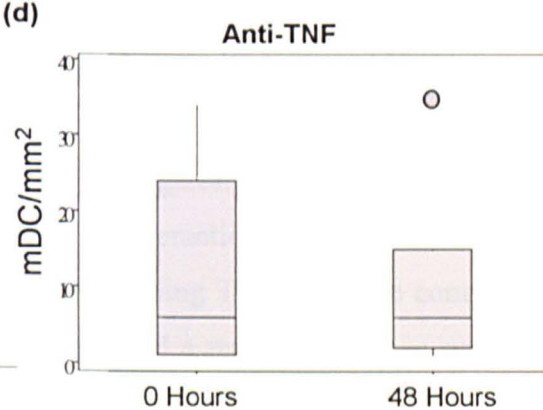
(b)



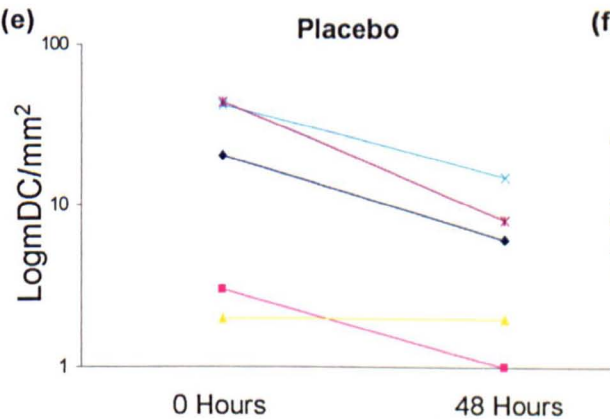
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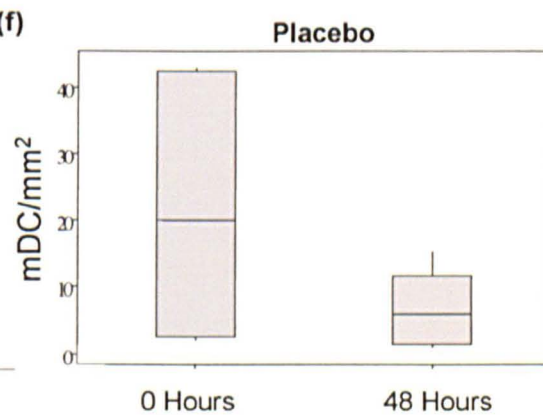
(d)



(e)



(f)



The pDC infiltrate to the inflamed synovium was greater in the placebo group compared to the infliximab group at baseline, but this was not statistically significant ( $p=0.3472$ ). Similar to mDC, a trend towards decreased pDC in SM was observed 48 hours after infliximab infusion, as compared to baseline, although this was not statistically significant (baseline, median = 2.7 (1-8.0) cells/mm<sup>2</sup>; 48 hours, median = 1.3 (0-3.0) cells/mm<sup>2</sup>,  $p=0.1102$ ) (Fig. 5.5c and 5.5d). Again, the number of pDC in SM of the placebo group also indicated a trend toward reduction at 48 hours after infusion compared to baseline, although this was not statistically significant (baseline, median = 7 (1-20) cells/mm<sup>2</sup>; 48 hours, median = 1.6 (1-3) cells/mm<sup>2</sup>,  $p=0.3472$ ) (Fig. 5.5e and 5.5f). As we observed in the RA study, in the PsA patient cohort the numbers of pDC in synovium were lower than we have observed in previous studies (chapter 4).

These analyses may suggest that TNF blockade may have differential effects on DC subsets. Given the rarity of this heterogeneous population and the relatively scarce number of patient samples available for study, identifying significant measurable alterations in SM after TNF blockade has proved difficult. As demonstrated in chapter 4, both mDC and pDC in synovium can be co-localised to T cell aggregates. We therefore chose to investigate the effects of anti-TNF- $\alpha$  on mDC and pDC interactions with T cells through *in vitro* methods. In particular we were interested in examining TNF blockade compared to IL-15 blockade as this laboratory has previously indicated a role for IL-15 in supporting macrophage/T cell contact induced TNF- $\alpha$  (630). These experiments will be discussed later (5.2.4). First however, we decided to elucidate further the broader biological mechanisms by which IL-15 neutralisation may operate, since the general effects of TNF blockade are comparatively well characterized. Specifically, we sought to characterize points by which IL-15 neutralisation may hinder T cell contact with DC.

### **5.3.2 IL-15 neutralisation study**

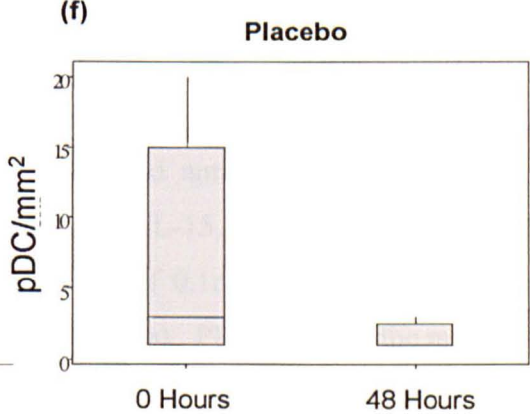
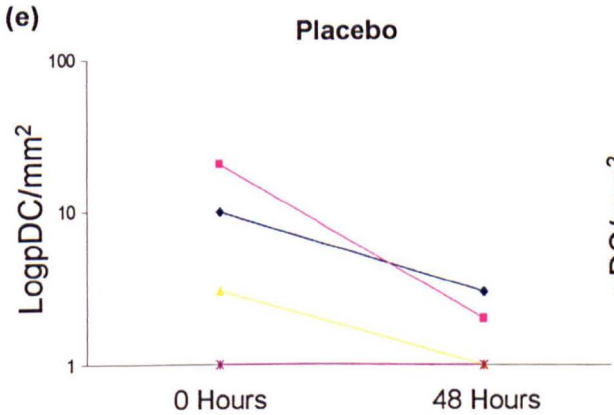
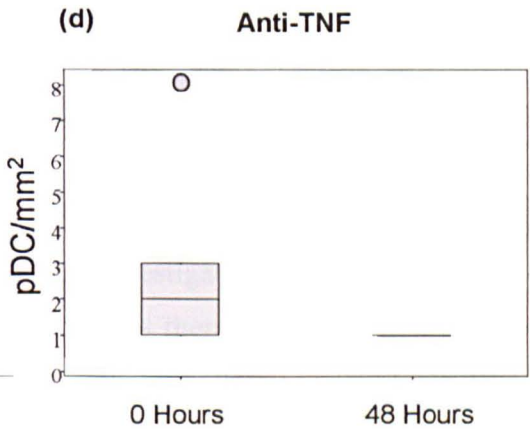
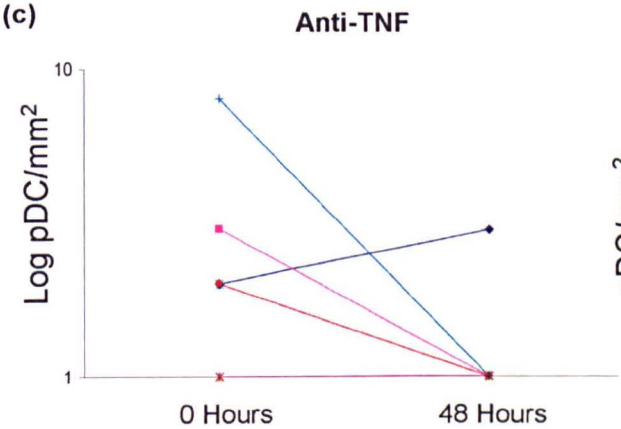
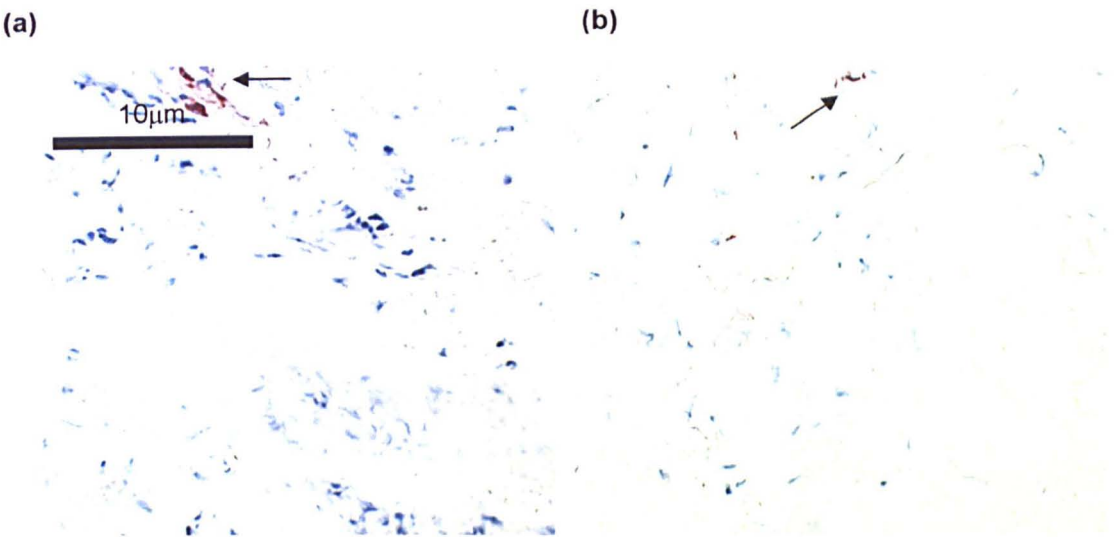
#### **5.3.2.1 Patients and cell lines**

##### **5.3.2.1.1 Patients**

Inflammatory arthritis patients attending Glasgow Royal Infirmary (GRI) fulfilled the American College of Rheumatology criteria for RA (23), or met diagnostic criteria for PsA

**Figure 5.5 CD304<sup>+</sup> pDC in paired PsA synovium before and 48 hours after infliximab or placebo infusion.**

The number of CD304<sup>+</sup> pDC infiltrating paired serial PsA SM biopsies taken before and 48 hours after treatment with infliximab (3mg/kg) (n=6) or placebo (n=6) was detected by immunohistochemical methods. CD304<sup>+</sup> mDC were visualized using DAB substrate (stained brown) and infiltration enumerated by computer assisted digital image analysis. One example of paired PsA SM showing CD304<sup>+</sup> pDC localized to the synovial sublining layer before (a) and 48 hours after (b) infliximab infusion is shown. In the infliximab group the overall trend was towards a decrease in the pDC infiltrate to synovium, although this did not attain statistical significance (c and d). In the placebo group the trend was towards a decrease in the pDC infiltrate (e and f). The graphs on the left show the total CD304<sup>+</sup> cells/mm<sup>2</sup> in each patient. The box and leaf plots on the right depict the median and interquartile range (50% of the data), flanked by whiskers indicating the extent of the entire patient group. Circles indicate outliers as determined using MiniTab.



as previously described (572, 573). All patients and healthy donors gave informed consent and the study protocol was approved by the Ethical Committee, GRI. Peripheral blood and synovial fluid was obtained from patients with RA (n=3) and peripheral blood was obtained from healthy controls (n=4) (Table 5.2). All cell analysis was undertaken on freshly isolated cells. For cytokine investigation, cell free SF and supernatants from cell culture were stored in the Centre for Rheumatic Disease (CRD) Biobank at -70°C until analysed. At the time of study, RA patients were receiving methotrexate (n=2) or sulphasalazine (n=1) or a combination of these agents (n=2). PsA patients were receiving either methotrexate (n=1) or sulphasalazine (n=3) or a combination of these agents (n=1). Patients on oral steroids were excluded from the study.

#### **5.3.2.1.2 BDB2 cell line**

BDB2 cells, an IL-15-dependent, human T cell line, were a kind gift of Dr. John Campbell, University of Glasgow.

#### **5.3.2.2 Biological action of anti-IL-15 antibodies**

The majority of both mDC and pDC localised to the synovial membrane express IL-15 (chapter 4). It was therefore of particular interest to investigate the possibility that IL-15 blockade in inflammatory arthritis may exert some of its therapeutic benefits by directly modifying DC. First however, we chose to elucidate the biological effects of anti-IL-15 in a panel of *in vitro* assays. For these analyses the IL-15-dependent, human T cell line BDB2 cells were utilised. Analysis of the bioactivities of anti-IL-15 were undertaken using F(ab')<sub>2</sub> fragmented anti-IL-15 in order to obviate Fc interactions (prepared as described in chapter 2).

##### **5.3.2.2.1 Assay of BDB2 cell death**

BDB2 cells are highly sensitive to IL-15 and its absence in culture leads to rapid BDB2 cell death. In order to determine if F(ab')<sub>2</sub> fragmented anti-IL-15 retained biological activity we analysed its ability to neutralise exogenous IL-15, causing BDB2 cell death. BDB2 cells were cultured in the presence or absence of 0.1ng/ml IL-15 and cell death analysed at 24 hours using propidium iodide (PI) (Fig. 5.6a). PI only stains the nucleus of



**Table 5.2.** Clinical features of RA and PsA patients and Healthy donors included in the study\*

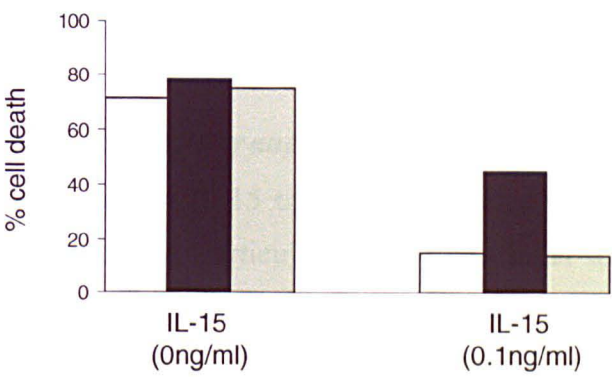
	Characteristic	PB study group	SF study group
RA	Age, Mean $\pm$ SD	58 $\pm$ 12 (24-80)	59 $\pm$ 15 (33-70)
	Female:Male	10:2	5:1
	WBC, Mean $\pm$ SEM	6.4x10 <sup>6</sup> /ml $\pm$ 1.2x10 <sup>4</sup> /ml	
	CRP (mg/liter)	26 $\pm$ 6 (6-100)	22 $\pm$ 7 (6-41)
PsA	Age, Mean $\pm$ SD	42 $\pm$ 14 (24-81)	41 $\pm$ 15 (25-50)
	Female:Male	10:3	5:1
	WBC, Mean $\pm$ SEM	8.0x10 <sup>6</sup> /ml* $\pm$ 4.5x10 <sup>5</sup> /ml	
	CRP (mg/liter)	14 $\pm$ 4 (6-73)	30 $\pm$ 15 (6-73)
Healthy	Age, Mean $\pm$ SD	44 $\pm$ 11	
	Female:Male	6:6	
	WBC, Mean $\pm$ SEM	6.4x10 <sup>6</sup> /ml $\pm$ 4.7x10 <sup>5</sup> /ml	

\*RA, rheumatoid arthritis; PsA, psoriatic arthritis; CRP, C reactive protein  
Mean  $\pm$  standard error of the mean (range) is shown.

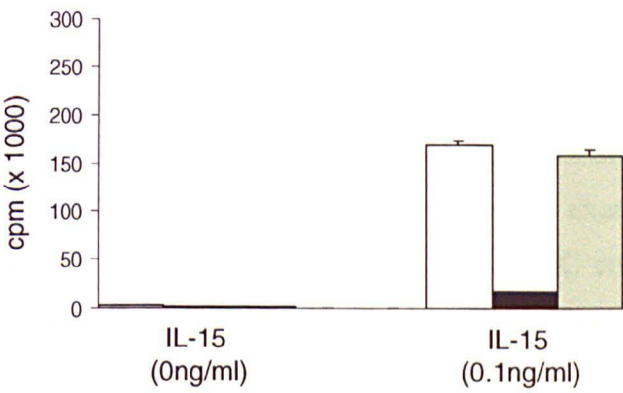
**Figure 5.6 Effects of anti-IL-15 F(ab')<sub>2</sub> on apoptotic rescue and proliferation of BDB2 cells.**

BDB2 cells were cultured with or without IL-15 (0.1ng/ml), in culture medium alone or supplemented with anti-IL-15 F(ab')<sub>2</sub> (10μg/ml) or human IgG (huIgG) F(ab')<sub>2</sub> (10μg/ml) negative control, and the percentage dead cells enumerated after 24 hours by propidium iodide staining and FACS analysis (a). In parallel experiments, BDB2 cells were cultured for 24 hours and proliferation was measured by determining <sup>3</sup>H-labeled thymidine uptake in the last 6 hours of culture (b). Values are the mean ± SEM of triplicate cultures. One example representative of three experiments is shown.

(a)



(b)



- medium alone
- anti-IL-15 F(ab')<sub>2</sub> (10μg/ml)
- hulgG F(ab')<sub>2</sub> (10μg/ml)

cells with ruptured membranes and therefore undergoing primary or secondary necrosis. In the absence of IL-15 over 70% of BDB2 cells were PI<sup>+</sup> (dead) after 24 hours, whereas only 14% of BDB2 were PI<sup>+</sup> in the presence of 0.1ng/ml IL-15. Addition of anti-IL-15 F(ab')<sub>2</sub>, but not irrelevant isotype IgG F(ab')<sub>2</sub>, induced cell death in 44.5% of BDB2 in IL-15 supplemented culture. The results were confirmed by parallel analysis of proliferation, as determined by <sup>3</sup>H-labeled thymidine uptake in the last 6 hours of culture (Fig 5.6b). Anti-IL-15 F(ab')<sub>2</sub>, but not irrelevant isotype IgG F(ab')<sub>2</sub>, suppressed BDB2 proliferation in 0.1ng/ml IL-15 supplemented cultures.

#### ***5.3.2.2.2 Inhibition of IL-15 induced IFN-γ and TNF-α release***

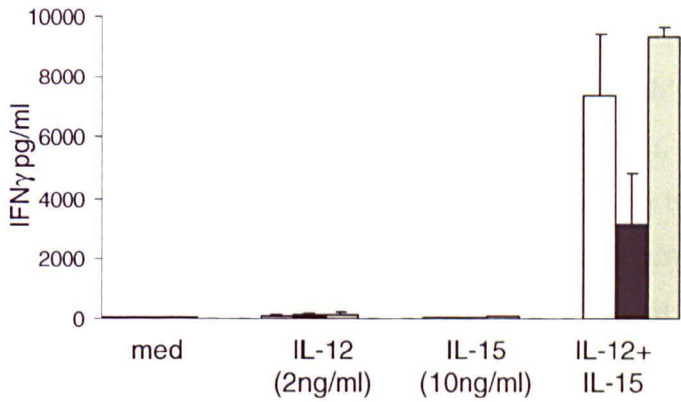
It has previously been established that IL-15 operates in synergy with IL-12 to promote cytokine production from lymphocytes, particularly IFN-γ. In order to assess biological activity of anti-IL-15 F(ab')<sub>2</sub> in a more relevant cell group – namely synovial fluid derived mononuclear cells, we analysed inhibition of IL-12/IL-15 induced IFN-γ release. Addition of anti-IL-15 F(ab')<sub>2</sub>, but not irrelevant isotype IgG F(ab')<sub>2</sub>, neutralised IL-12/IL-15 induced IFN-γ release from SF derived mononuclear cells (SFMC) as determined by ELISA (Fig. 5.7a). This assay confirms the bioactivity of anti-IL-15.

It has previously been shown that RA SFMC are capable of spontaneous TNF-α release, which can be enhanced by addition of IL-15. We therefore chose to examine the effect of endogenous IL-15 neutralization on TNF-α release from RA SFMC culture. TNF-α in culture supernatants was measured by ELISA after 16 hours. As expected, addition of exogenous IL-15 increased TNF-α release and this effect was abrogated by neutralization using anti-IL-15 F(ab')<sub>2</sub>. Of interest, the spontaneous release of TNF-α from the SF derived cells was reduced by the addition of anti-IL-15 F(ab')<sub>2</sub> (Fig. 5.7b). Isotype control did not affect TNF-α levels. Together these data demonstrate that neutralisation of IL-15 can have down-stream effects on other inflammatory pathways and TNF in particular. Furthermore, the evidence suggests blockade of endogenous IL-15, indicating that leukocytes originating from the inflamed synovial environment are sensitive to IL-15 neutralisation.

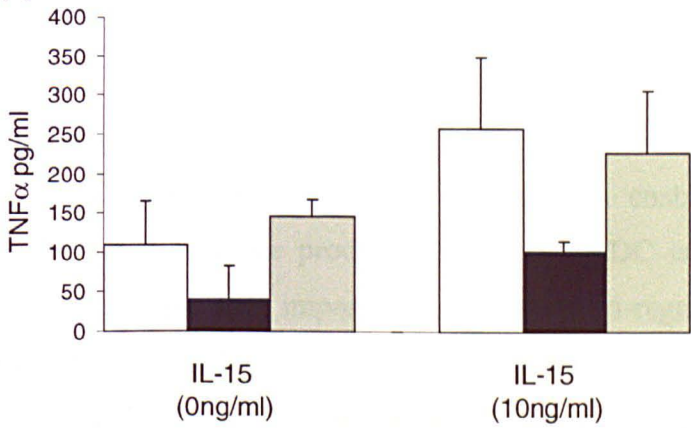
**Figure 5.7 Effects of anti-IL-15 F(ab')<sub>2</sub> on cytokine production from RA synovial fluid mononuclear cells.**

Rheumatoid arthritis (RA) synovial fluid mononuclear cells (SFMC) were incubated for 16 hours in RPMI/10% foetal calf serum (FCS) with or without the cytokines as shown (2ng/ml IL-12, 10ng/ml IL-15) in the presence or absence of anti-IL-15 F(ab')<sub>2</sub> (10µg/ml) or human IgG (huIgG) F(ab')<sub>2</sub> (10µg/ml) negative control, and interferon-γ (IFN-γ) levels in culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA) (a). In parallel experiments RA SFMC were cultured for 16 hours in RPMI/10% FCS with or without IL-15 (10ng/ml), in the presence or absence of anti-IL-15 F(ab')<sub>2</sub> (10µg/ml) or human IgG (huIgG) F(ab')<sub>2</sub> (10µg/ml) negative control, and TNF-α in culture supernatant measured by ELISA (b). Values are the mean ± SEM of triplicate cultures. Similar data was obtained from 4 RA patients.

(a)



(b)



medium alone  
anti-IL-15 F(ab')<sub>2</sub> (10 $\mu$ g/ml)  
huIgG F(ab')<sub>2</sub> (10 $\mu$ g/ml)

#### ***5.3.2.2.3 Down-regulation of surface CD69***

We and others have previously shown that IL-15 enhances CD69 expression on PB and SF derived T cells. CD69 is, in turn, implicated in the cognate interactions between T cells and APC, and has been shown to be upregulated on cells within the context of active inflammatory arthritis (631, 671). In order to elucidate the mechanisms by which IL-15 blockade may inhibit these cognate interactions, specifically those between T cells and DC, we chose to analyse CD69 expression on RA PBMC and RA SFMC. First, to study exogenous IL-15 induced up-regulation of surface CD69, cells were rested overnight before 24 hour incubation with or without IL-15 and anti-IL-15 F(ab')<sub>2</sub> or irrelevant isotype IgG F(ab')<sub>2</sub>. Consistent with the above data, anti-IL-15 F(ab')<sub>2</sub>, but not isotype IgG F(ab')<sub>2</sub>, neutralized exogenous IL-15 induced CD69 upregulation on both RA PB and SF derived mononuclear cells (Fig. 5.8a). In addition, parallel experiments examining freshly isolated RA PB and SF mononuclear cells (in the absence of added IL-15) revealed that CD69 expression was reduced by addition of anti-IL-15 F(ab')<sub>2</sub>, but not isotype IgG F(ab')<sub>2</sub> (Fig. 5.8b). The data therefore confirm neutralisation of endogenous IL-15 and indicate a mechanism by which IL-15 neutralization could inhibit cognate DC/T cell contact.

#### ***5.3.2.2.4 Binding of membrane bound IL-15***

The initial release of IL-2 by DC (both pDC and mDC) is required to enable DC directed clonal expansion of T cells. However, the production of IL-2 by DC is itself initially triggered by T-cell mediated stimuli and, importantly, is tightly co-regulated with the expression of IL-15. Indeed, IL-2 production by DC in IL-15-deficient mice is strongly impaired (647). The down-regulation of CD69 on the T cell is one mechanism by which this phenomenon could be explained. We sought to examine if anti-IL-15 could impair cell contact directly by binding surface bound IL-15 as analysed by FACs methodology.

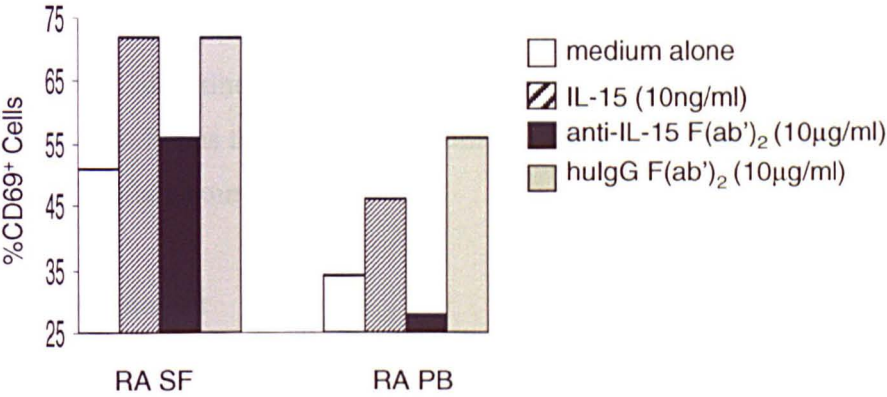
IL-15 binds at the cell surface to a heterotrimeric IL-15 receptor composed of IL-15R $\alpha$ , IL-15/2R $\beta$  and common  $\gamma$  chain (672, 673). Analyses of IL-15 binding to the IL-15 receptor complex has been challenging as the receptor is expressed at only low levels on primary cells. Previous groups have therefore produced IL-15 transfected cell lines to allow detection of surface bound IL-15 (674). Because BDB2 are particularly sensitive to IL-15, we first analysed expression of IL-15R $\alpha$  in order to determine if expression was

**Figure 5.8 Effects of anti-IL-15 F(ab')<sub>2</sub> on IL-15 dependent CD69 expression on RA synovial fluid mononuclear cells.**

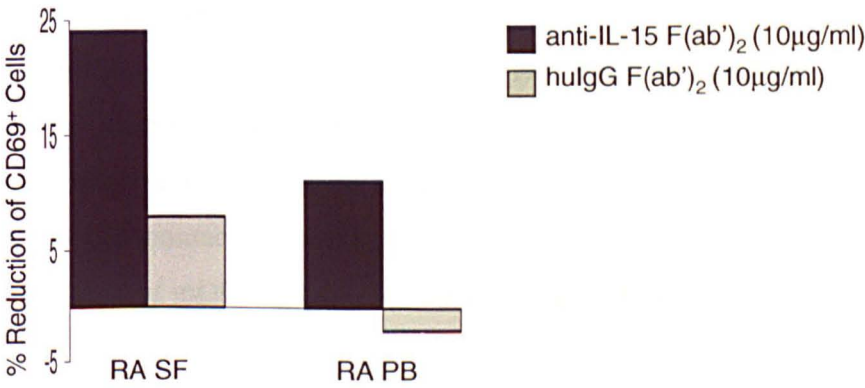
RA peripheral blood mononuclear cells (PBMC) or synovial fluid mononuclear cells (SFMC) were rested overnight after purification, then incubated for 24 hours with or without IL-15 (10ng/ml) in the presence of absence of anti-IL-15 F(ab')<sub>2</sub> (10μg/ml) or human IgG (huIgG) F(ab')<sub>2</sub> (10μg/ml) negative control, prior to fluorescence-activated cell sorting for CD69 expression (percentage of CD69<sup>+</sup> cells within the lymphocyte region) (a). Data are representative of 6 similar experiments. In parallel experiments to determine the activity of anti-IL-15 F(ab')<sub>2</sub> without addition of exogenous IL-15, the percentage of CD69<sup>+</sup> lymphocytes on freshly isolated RA PBMC and RA SFMC after incubation with anti-IL-15 F(ab')<sub>2</sub> (10μg/ml) or human IgG (huIgG) F(ab')<sub>2</sub> (10μg/ml) negative control was calculated. The %reduction of CD69<sup>+</sup> lymphocytes is shown. Data are representative of 6 similar experiments.



(a)



(b)



sufficiently high to enable FACS detection, and by association, detection of receptor-bound IL-15. The level of surface IL-15R $\alpha$  on BDB2 cells was determined by FACS analysis using the IL-15R $\alpha$  specific antibody M161. BDB2 cells express a high level of surface IL-15R $\alpha$ , detectable by FACS analysis, and consistent with their sensitivity to IL-15 in culture (Fig. 5.9a). Furthermore, IL-15R $\alpha$  binding by M161 could be inhibited by pre-incubating BDB2 cells in exogenous IL-15 for 30 minutes prior to staining (Fig. 5.9b). We therefore concluded that BDB2 surface IL-15 receptor expression was sufficiently high for FACS and transfection was not required. We next incubated BDB2 cells in IL-15 for 30 minutes before addition of biotin-conjugated anti-IL-15 F(ab')<sub>2</sub> for 30 minutes followed by streptavidin-FITC. Anti-IL-15 F(ab')<sub>2</sub>, but not isotype control IgG F(ab')<sub>2</sub>, bound membrane bound IL-15, as determined by FACS analysis (Fig. 5.9c). These data suggest that IL-15 neutralising antibody has the potential to inhibit cognate cell contact enabled by paracrine presentation of surface bound IL-15.

### 5.3.3 Dendritic cell-T cell contact

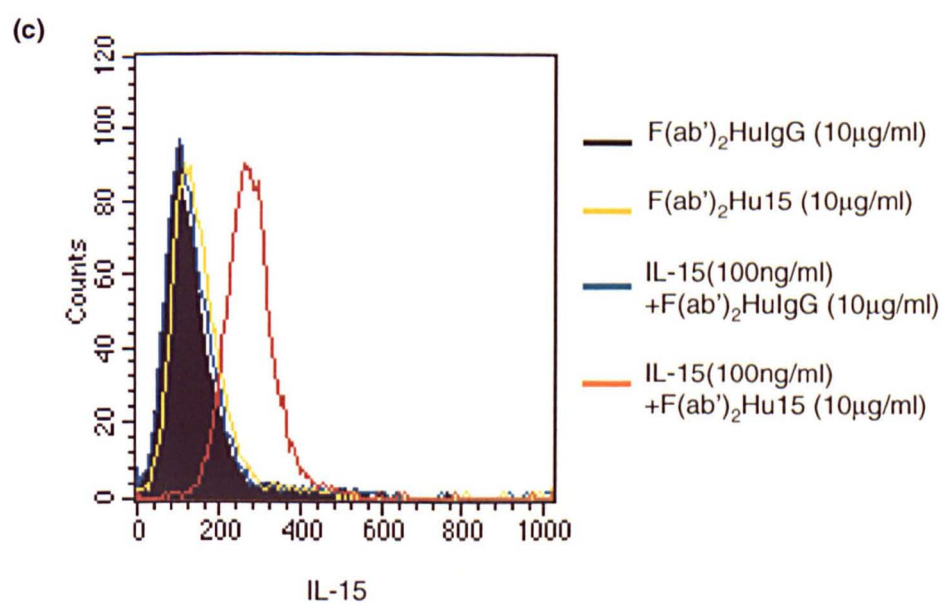
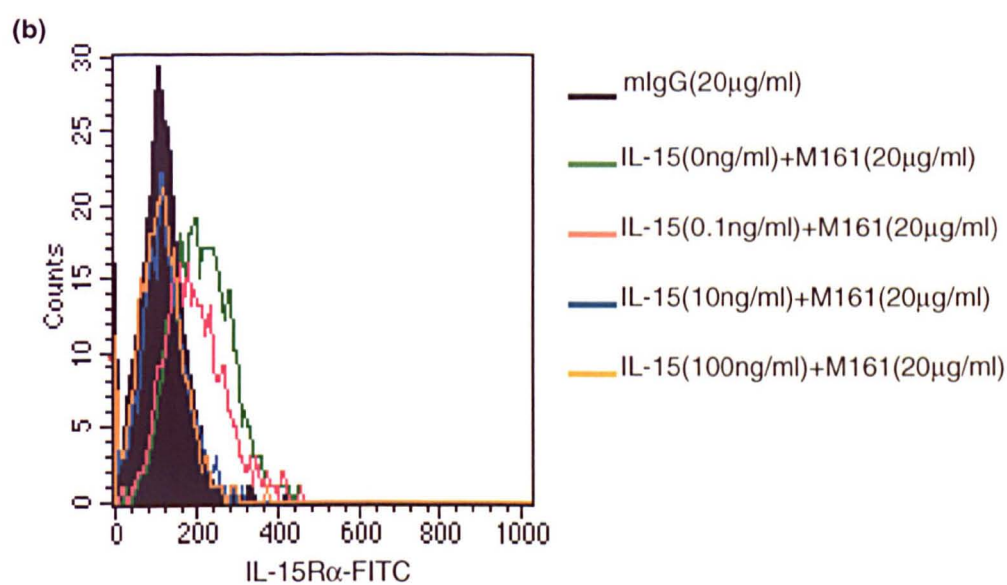
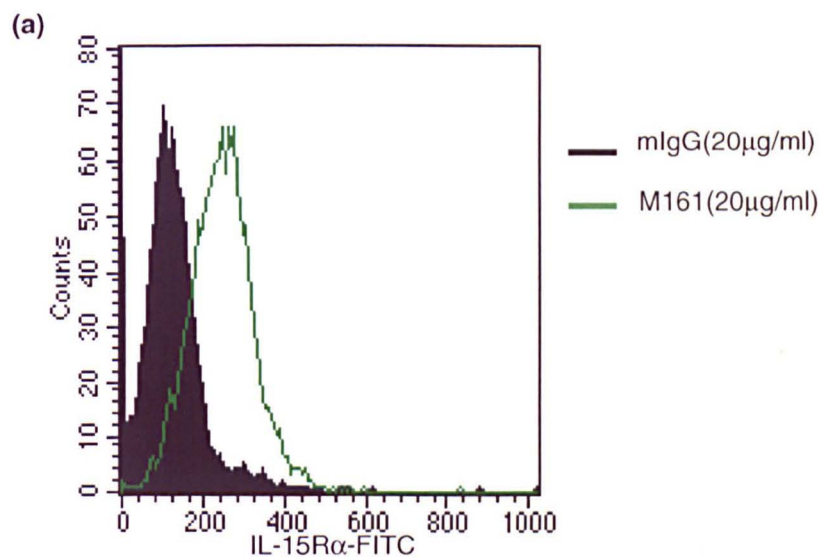
T cells are in close association with APC in the inflamed synovium (675). This provides anatomical rationale whereby *in vivo* APC/T cell contact could be of importance in disease pathogenesis, particularly as in driving the release of pro-inflammatory cytokines such as TNF- $\alpha$  (676). Direct cell-contact-mediated interactions have been the focus of several *in vitro* studies aimed at replicating this *in vivo* environment. However, the majority of these studies have focused on monocyte/macrophage-T cell interactions (677, 678). As described in chapter 4, mDC and pDC are in close contact with T cells in synovium, commensurate with *in situ* cell-contact. However no studies thus far have examined, by *in vitro* methods, the interactions of mDC and pDC with pre-activated T cells.

#### 5.3.3.1 Interactions between DC and T cells

We first needed to develop an assay by which to study mDC/pDC-T cell contact *in vitro* (Fig. 5.10). The majority of mDC and pDC in the synovium are immature, but T cells display an activated phenotype. For pragmatic reasons we chose to examine the effect of pre-activated T cells on immature mDC and pDC purified directly from blood. For these experiments healthy donor PB was used as RA and PsA PB had insufficient obtainable DC numbers (as discussed in chapter 3). CD3<sup>+</sup> T cells were purified by positive selection from

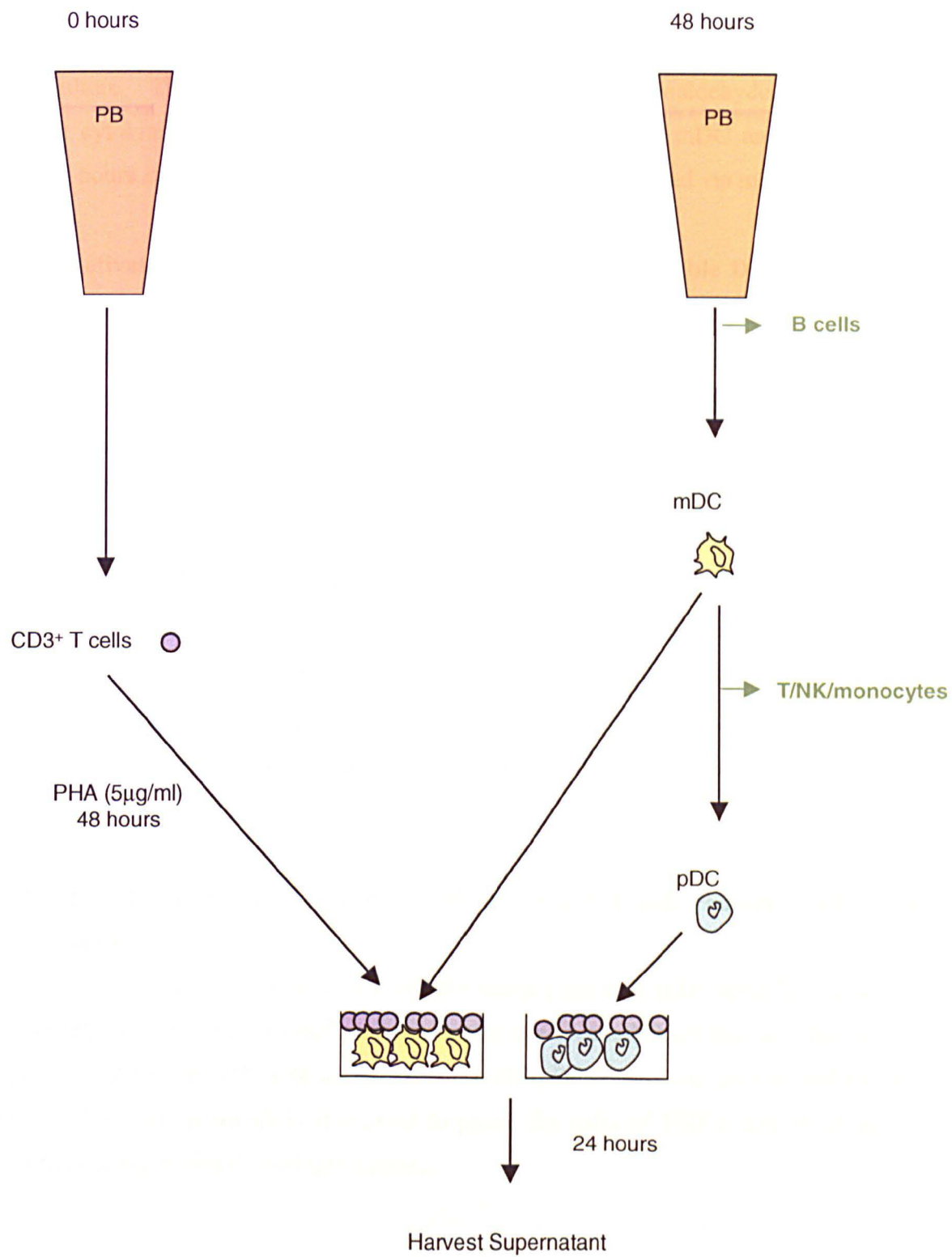
**Figure 5.9 The expression of IL-15R $\alpha$  on BDB2 cells and the capacity of anti-IL-15 F(ab')<sub>2</sub> to bind membrane bound IL-15.**

The expression levels of IL-15-R $\alpha$  on BDB2 cells was detected by incubation with IL-15R $\alpha$  specific detection antibody, M161, followed by detection with goat-anti-mouse-IgG-FITC and fluorescence-activated cells sorting (FACS) analysis (a). The binding of M161 to IL-15R $\alpha$  was inhibited by pre-incubating cells with exogenous IL-15 (0.1 – 100ng/ml) for 30 minutes before staining (b). To evaluate ability of anti-IL-15 F(ab')<sub>2</sub> to bind membrane bound IL-15, BDB2 cells were first incubated with or without IL-15 (100ng/ml) for 30 minutes, followed by incubation with anti-IL-15 F(ab')<sub>2</sub> (10 $\mu$ g/ml) or human IgG (huIgG) F(ab')<sub>2</sub> (10 $\mu$ g/ml) negative control, and detection with goat-anti-human IgG (Fab) and FACS analysis (c). One example of 5 similar experiments is shown.



**Figure 5.10 Design of an *in vitro* experimental model of mDC and pDC contact with blasted T cells.**

CD3<sup>+</sup> T cells were purified by positive magnetic cell sorting from peripheral blood (PB) from a healthy donor and blasted by incubation in phytohaemagglutinin (5µg/ml) for 48 hours, after which time they were either fixed for 2 hours in PBS/2%paraformaldehyde, or not. At 48 hours CD1c<sup>+</sup>/CD11c<sup>+</sup> mDC were purified by positive magnetic cell sorting and CD303<sup>+</sup>/CD123<sup>+</sup> pDC were purified by negative magnetic cell sorting from PB from the same donor. mDC (culture supplemented with GM-CSF) and pDC (culture supplemented with IL-3) were co-cultured with T cells at a ratio of 3:1 (T cells:DC) and supernatant harvested at 24 hours.



PB and blasted by 48 hour culture with phytohaemagglutinin (PHA) (5 $\mu$ g/ml), in order to produce an activated phenotype similar to that displayed in synovium (676). Purity was routinely >98% (Fig. 5.11c). At 48 hours, mDC and pDC were sequentially purified by positive (mDC) and negative (pDC) selection from freshly obtained autologous PB, with purity routinely >98% (pDC) (Fig. 5.11a) and >96% (mDC) (Fig. 5.11b). This was important to ensure DC subsets were as immature as possible, since DC mature rapidly upon culture. The blast T cells were either fixed in paraformaldehyde (thus only DC released cytokines were measured), or not, and added back to mDC and pDC cultures. After 24 hours supernatants were harvested and cytokines measured via multiplex analysis.

#### **5.3.3.2 Activated T cells and cell contact are required to enable DC/T cell induced TNF- $\alpha$ production**

We first analysed TNF- $\alpha$  release following the addition of paraformaldehyde fixed or live, non-PHA blasted T cells (resting), to mDC or pDC cultures. As expected, resting T cells induce negligible levels of TNF- $\alpha$  in culture with mDC or pDC, regardless of fixation (Fig. 5.12a and c) or live cell culture (Fig. 5.12b and d). Activated T cells induced TNF release in contact with DC most likely through ligand interactions including CD69/CD69R, CD40/CD40L, TcR/HLA-DR and CD28/CD80-CD86. We confirmed that cell contact was required to induce appreciable TNF- $\alpha$  release from mDC/pDC in culture with PHA blasted T cells. When a cell membrane insert was used to separate T cells from the DC, TNF- $\alpha$  in culture was markedly reduced in mDC/T cell culture (Fig. 5.13a) and pDC/T cell culture (Fig. 5.13b). Therefore the TNF- $\alpha$  released by the DC subsets could not be attributed to soluble factors alone.

#### **5.3.3.3 Effects of anti-TNF- $\alpha$ and anti-IL-15 on DC/T cell contact cytokine and chemokine release**

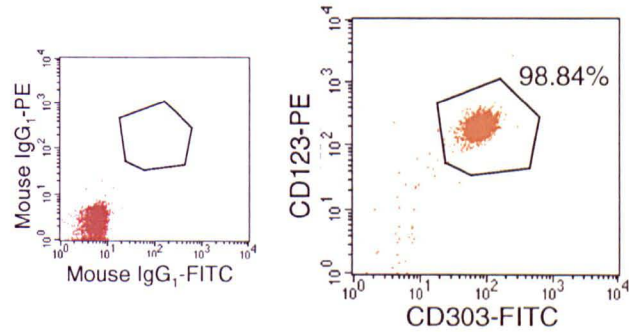
Since IL-15 could play a role in T cell cognate interactions with mDC and pDC via indirect (CD69 regulation) or direct (surface-bound paracrine induced IL-2) mechanisms, we next chose to evaluate the effect of anti-IL-15 on mDC/pDC-T cell contact induced cytokine release. We were particularly interested to probe the roles of TNF- $\alpha$  and IL-15 in such conditions using available biologic agents.

### **Figure 5.11 Purity of isolated pDC, mDC and CD3<sup>+</sup> T cell**

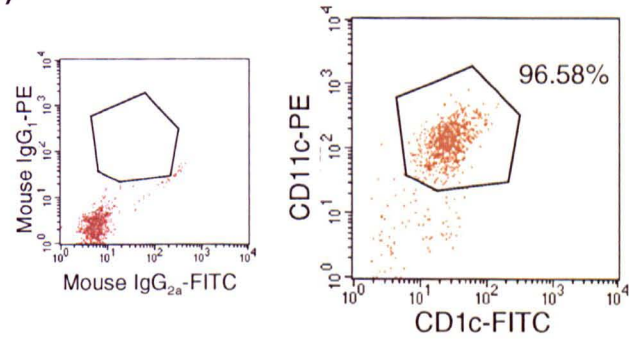
The purity of PB isolated mDC, pDC and CD3 was determined by staining with CD303/CD123 (pDC) (a), CD1c/CD11c (mDC) (b), CD3 (T cells) (c) or appropriate isotype controls following by fluorescence-activated cells sorting (FACS) analysis. Purity of mDC was routinely over 96% for mDC, over 98% for pDC and over 97% for CD3. One example is shown of each.



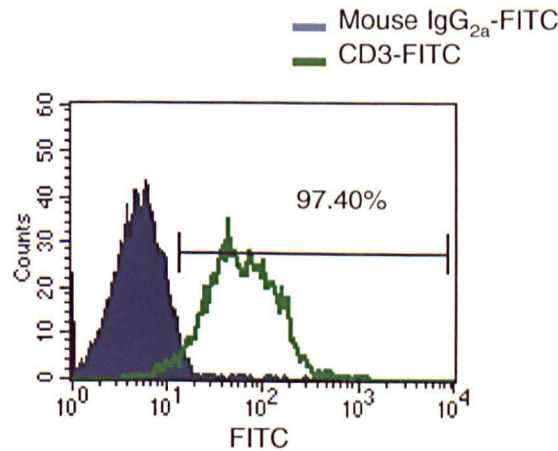
(a)



(b)

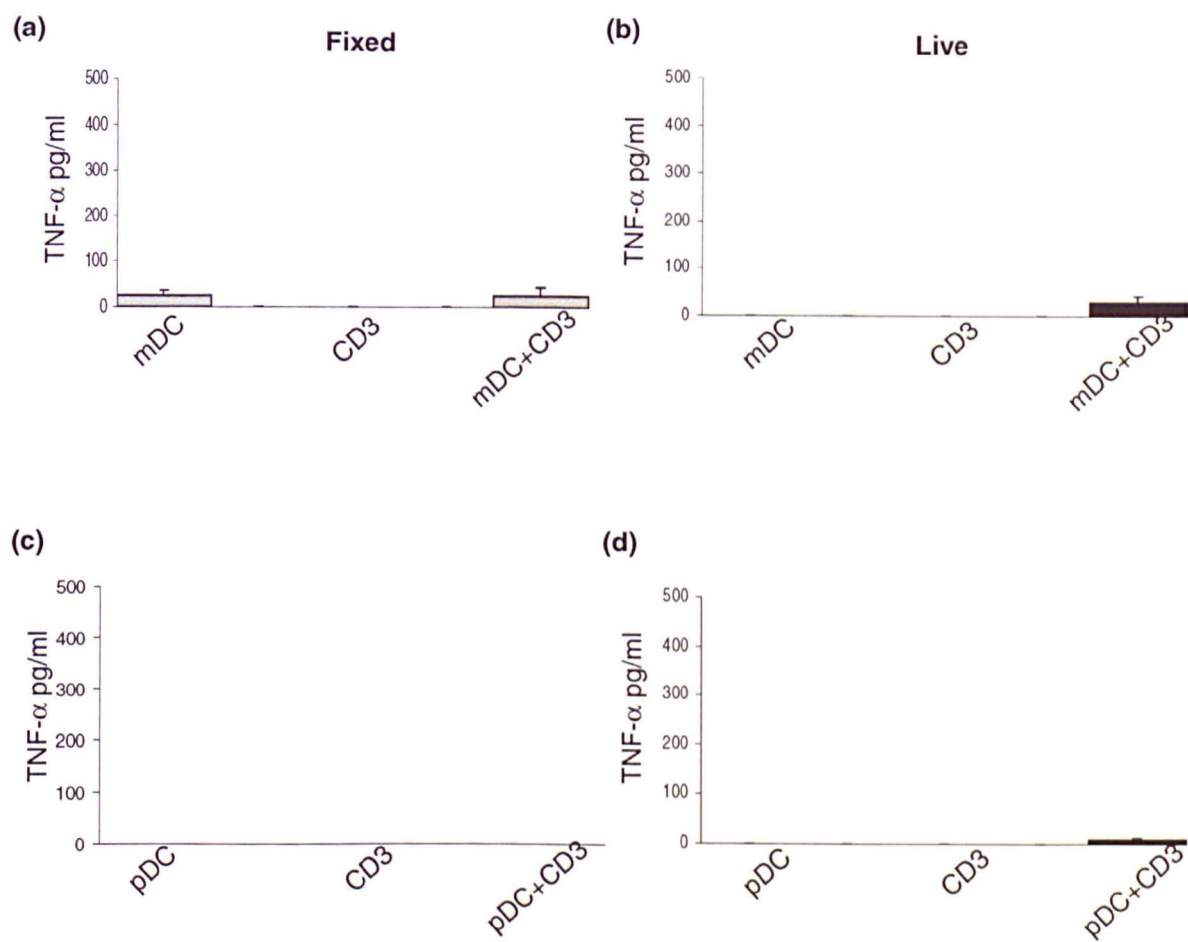


(c)



**Figure 5.12 T cell activation is required to enable cell contact dependent TNF- $\alpha$  release from mDC and pDC.**

TNF- $\alpha$  levels in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) after 24 hours of mDC co-culture with resting CD3<sup>+</sup> T cells that had been fixed in PBS/2%paraformaldehyde (Fixed) (a) or not (Live) (b) and pDC co-culture with resting CD3<sup>+</sup> T cells that had been fixed in PBS/2%paraformaldehyde (Fixed) (c) or not (Live) (d). Values are the mean  $\pm$  SEM of triplicate cultures. Similar data was obtained from 2 healthy donors.

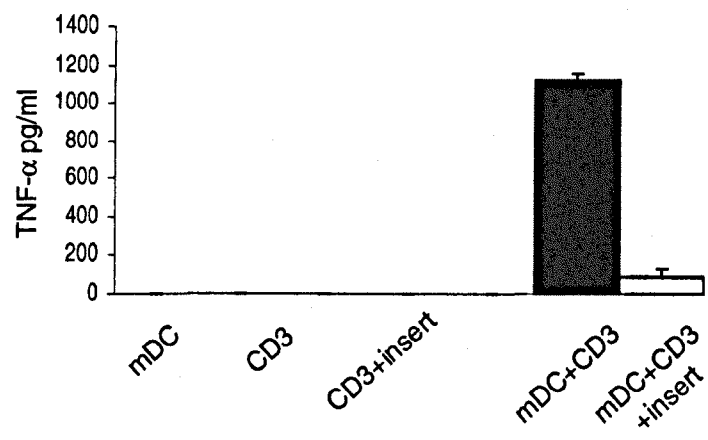


**Figure 5.13 Cell contact is required to allow activated T cell induction of TNF- $\alpha$  from mDC and pDC.**

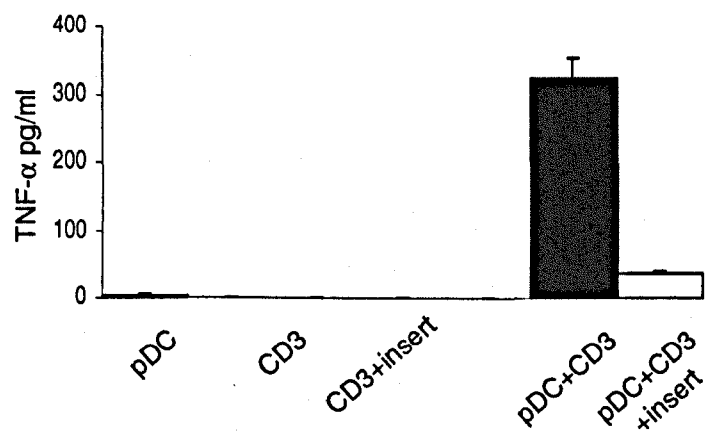
Prior to co-culture, CD3<sup>+</sup> T cells from healthy donor peripheral blood (PB) were incubated in the presence of PHA (5 $\mu$ g/ml) for 48 hours. The activated T cells (Live) were then co-cultured in direct contact to, or separated by a permeable cell membrane insert with, either mDC (a) or pDC (b), freshly isolated by magnetic cell sorting from the same donor. TNF- $\alpha$  levels in culture supernatant were measured at 24 hours by enzyme-linked immunosorbent assay (ELISA) at 24 hours. Values are the mean  $\pm$  SEM of triplicate cultures. Similar data was obtained from 2 healthy donors.

(a)

Live



(b)



For these experiments mouse IgG<sub>1</sub> anti-human TNF- $\alpha$  and mouse IgG<sub>1</sub> anti-human IL-15 were utilised for a number of reasons. First, technical difficulties precluded the F(ab')<sub>2</sub> fragmentation of infliximab or etanercept. Second, due to low total numbers of mDC and pDC that could be purified from blood and to allow comparative analysis, the same isotype control was needed for both anti-TNF- $\alpha$  and anti-IL-15. Because the isotype control for infliximab is human IgG, anti-IL-15 (Fab')<sub>2</sub> could therefore not be used. Third, human IgG itself is highly anti-inflammatory via the Fc $\gamma$ RIIa receptor, so would obscure any effects that either anti-TNF- $\alpha$  or anti-IL-15 might have. Therefore murine anti-human antibodies were used to obviate these problems.

#### **5.3.3.3.1 TNF- $\alpha$**

Multiplex analysis of culture supernatants demonstrated, as expected, that anti-TNF- $\alpha$  neutralised TNF- $\alpha$  released by pDC and mDC co-cultured with either fixed (Fig. 5.14a and c respectively) or live (Fig. 5.14b and d respectively) PHA blasted T cells ( $p < 0.0001$ ). In contrast, anti-IL-15 did not significantly reduce TNF- $\alpha$  in mDC or pDC cultures with fixed T cells. However, anti-IL-15 did significantly reduce TNF- $\alpha$  levels in live T cell cultures (mDC,  $p = 0.008$ , pDC  $p = 0.009$ ). Mouse IgG<sub>1</sub> isotype control did not alter TNF- $\alpha$  levels in fixed or live cultures. These data are consistent with the model whereby IL-15 enables/enhances DC-T cell contact driven T cell promotion of DC cytokine release. Clearly such a mechanism would not operate in fixed T cell cultures.

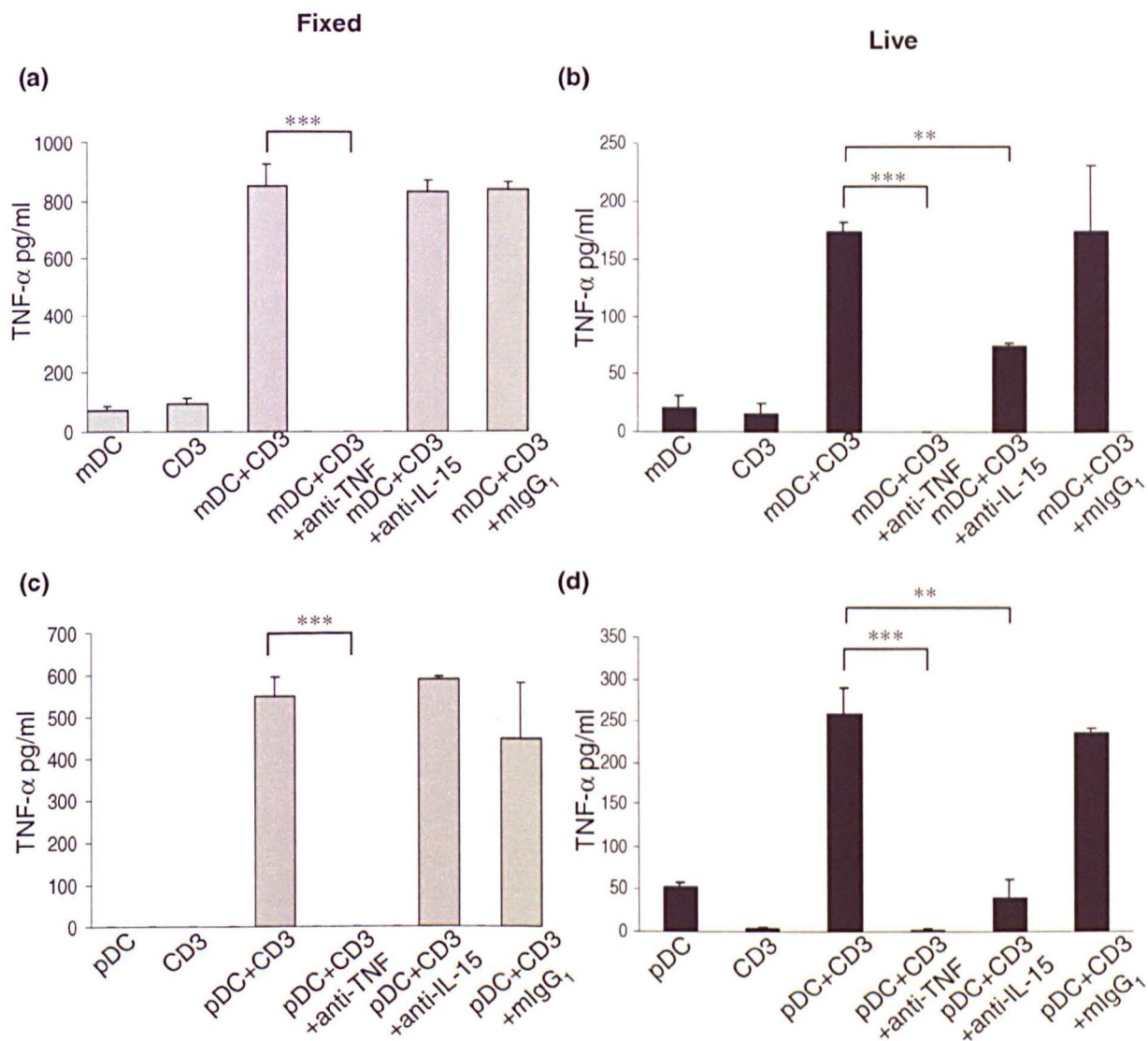
#### **5.3.3.3.2 CCL3**

CCL3, formerly designated macrophage inflammatory protein-1- $\alpha$  (MIP-1 $\alpha$ ), was measurable in supernatants of fixed and live mDC/T cell cultures and fixed and two of three live pDC/T cell cultures, although at lower levels. Neither anti-TNF- $\alpha$ , nor anti-IL-15 significantly reduced CCL3 in mDC or pDC co-cultures with fixed T cells (Fig. 5.15a and c). However, in live T cell co-culture with mDC both anti-TNF- $\alpha$  and anti-IL-15 reduced CCL3 levels (significant for anti-IL-15,  $p = 0.049$ ) (Fig. 5.15b). In contrast, in two of three pDC co-cultures with live T cells anti-TNF- $\alpha$ , but not anti-IL-15, increased the amount of CCL3 released ( $p = 0.029$ ) (Fig. 5.15d). CCL3 was not measurable in culture supernatants from the third donor. Mouse IgG<sub>1</sub> isotype control did not alter CCL3 levels in fixed or live cultures. These results were consistent with the analysis of infliximab effect

**Figure 5.14 TNF- $\alpha$  production in cultures of mDC and pDC with fixed or live CD3<sup>+</sup> T cells.**

Prior to co-culture, CD3<sup>+</sup> T cells from healthy donor peripheral blood (PB) were incubated in the presence of PHA (5 $\mu$ g/ml) for 48 hours. In half of experiments as denoted, T cells were then fixed in PBS/2%paraformaldehyde (Fixed) or left alone (Live). At 48 hours mDC and pDC were sequentially purified by magnetic cell sorting from the same donor. mDC and fixed (a) or live (b) T cells were cultured alone, or together at a ratio of 3:1(T cells:mDC) in the presence or absence of anti-TNF- $\alpha$  (100ng/ml), anti-IL-15 (100ng/ml) or mouse (m) IgG<sub>1</sub> (100ng/ml) negative control. In parallel assays, pDC and fixed (c) or live (d) T cells were cultured alone, or together at a ratio of 3:1(T cells:mDC) in the presence or absence of anti-TNF- $\alpha$  (100ng/ml), anti-IL-15 (100ng/ml) or mouse (m) IgG<sub>1</sub> (100ng/ml) negative control. TNF- $\alpha$  levels in culture supernatant were measured at 24 hours by multiplex analysis at 24 hours. Values are the mean  $\pm$  SEM of triplicate cultures. Similar data was obtained from 6 healthy donors.

\*\*p<0.01, \*\*\*p<0.0001



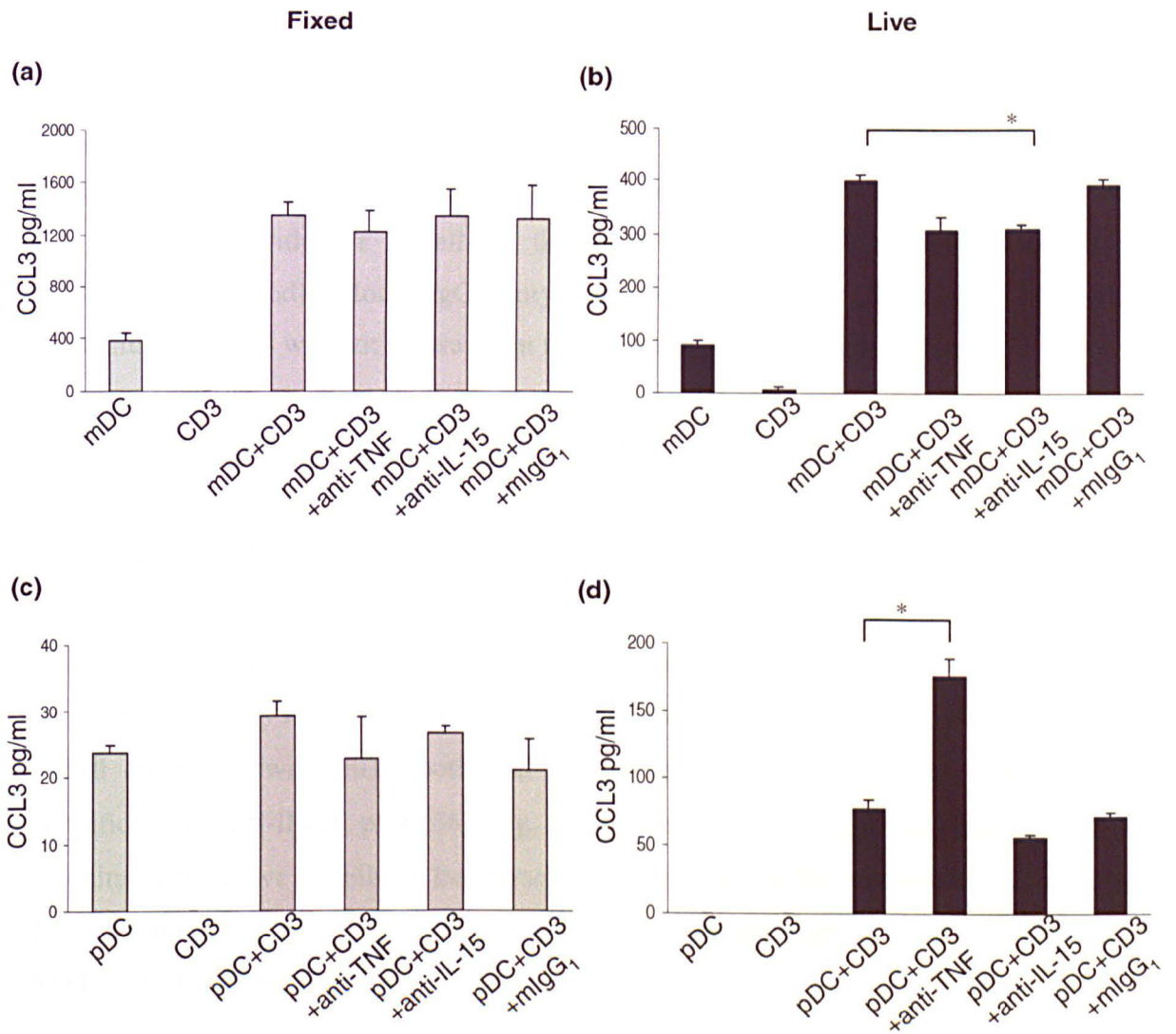
Anti-TNF $\alpha$  = 100ng/ml  
 Anti-IL-15 = 100ng/ml  
 Mouse IgG<sub>1</sub> = 100ng/ml



**Figure 5.15 CCL3 production in cultures of mDC and pDC with fixed or live CD3<sup>+</sup> T cells.**

Prior to co-culture, CD3<sup>+</sup> T cells from healthy donor peripheral blood (PB) were incubated in the presence of PHA (5μg/ml) for 48 hours. In half of experiments as denoted, T cells were then fixed in PBS/2%paraformaldehyde (Fixed) or left alone (Live). At 48 hours mDC and pDC were sequentially purified by magnetic cell sorting from the same donor. mDC and fixed (a) or live (b) T cells were cultured alone, or together at a ratio of 3:1(T cells:mDC) in the presence or absence of anti-TNF-α (100ng/ml), anti-IL-15 (100ng/ml) or mouse (m) IgG<sub>1</sub> (100ng/ml) negative control. In parallel assays, pDC and fixed (c) or live (d) T cells were cultured alone, or together at a ratio of 3:1(T cells:mDC) in the presence or absence of anti-TNF-α (100ng/ml), anti-IL-15 (100ng/ml) or mouse (m) IgG<sub>1</sub> (100ng/ml) negative control. CCL3 levels in culture supernatant were measured at 24 hours by multiplex analysis at 24 hours. Values are the mean ± SEM of triplicate cultures. Similar data was obtained from 6 healthy donors.

\*p<0.05



Anti-TNF $\alpha$  = 100ng/ml  
Anti-IL-15 = 100ng/ml  
Mouse IgG<sub>1</sub> = 100ng/ml

on DC subsets in synovium discussed earlier in this chapter, which suggested that TNF blockade may have differential effects on DC migration.

#### **5.3.3.3.3 CCL4**

CCL4, formerly designated MIP-1 $\beta$ , was measurable in supernatants of fixed and live mDC/T cell cultures and fixed and live pDC/T cell cultures, but again pDC levels were lower than those measured from mDC. Similar to CCL3, anti-TNF- $\alpha$  and anti-IL-15 did not significantly reduce CCL4 in fixed cell cultures (Fig. 5.16a and c). However, in live T cell co-culture with mDC both anti-TNF- $\alpha$  and anti-IL-15 reduced CCL4 levels (significant for anti-TNF- $\alpha$ ,  $p=0.045$ ) (Fig. 5.16b). As before, CCL4 was increased in two of three pDC co-cultures with live T cells in the presence of anti-TNF- $\alpha$ , but not anti-IL-15 ( $p=0.033$ ) (Fig. 5.16d). Mouse IgG<sub>1</sub> isotype control did not alter CCL4 levels in fixed or live cultures. CCL4 was not measured in the supernatant from the third donor.

#### **5.3.3.3.4 CXCL8**

CXCL8, formerly designated IL-8, was measurable in supernatants of fixed and live mDC/T cell cultures and fixed and live pDC/T cell cultures, but again pDC levels were much lower than those measured from mDC. In addition mDC alone were capable of high CXCL8 release, although this was enhanced by T cell contact. Anti-TNF- $\alpha$  and anti-IL-15 did not significantly reduce IL-8 in fixed cell cultures (Fig. 5.17a and c). However, in live T cell co-culture with mDC both anti-TNF- $\alpha$  and anti-IL-15 reduced CXCL8 levels (significant for anti-IL-15,  $p=0.036$ ) (Fig. 5.17b). As before CXCL8 was increased in pDC co-cultures with live T cells in the presence of anti-TNF- $\alpha$ , but not anti-IL-15, although this was not significant (Fig. 5.17d). Mouse IgG<sub>1</sub> isotype control did not alter CXCL8 levels in fixed or live cultures.

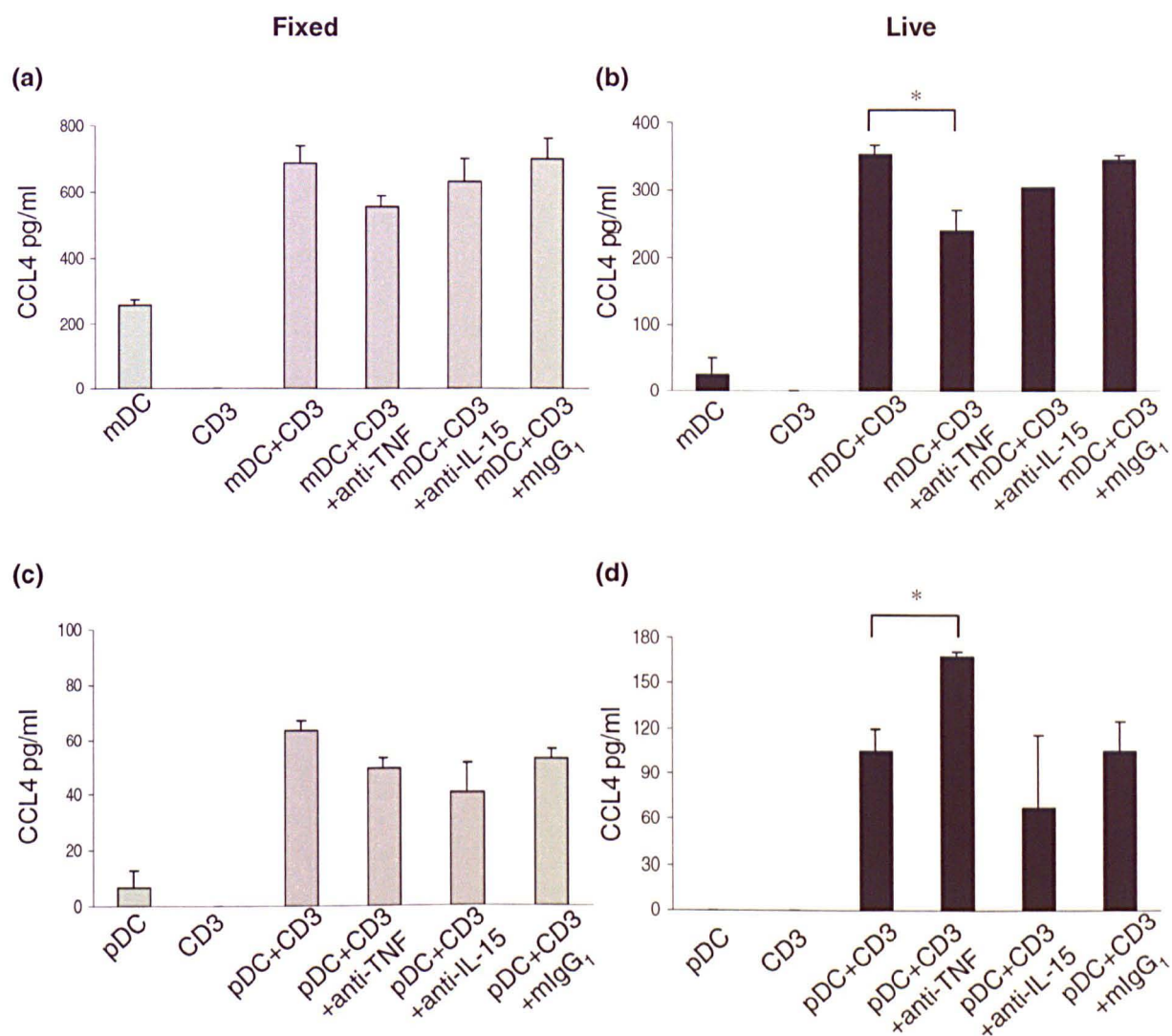
#### **5.3.3.3.5 IFN- $\alpha$**

The increase in chemokine levels in pDC-T cell cultures induced by anti-TNF- $\alpha$ , but not anti-IL-15, was intriguing. Recent studies have demonstrated that IFN- $\alpha$  specifically triggers CCL2, CCL3 and CCL4 secretion by pDC, but not mDC (679). Furthermore, it has been hypothesised that TNF- $\alpha$  and IFN- $\alpha$  may counter-balance each other, with the former inhibiting secretion of the latter from pDC (591). Since pDC are the major type I

**Figure 5.16 CCL4 production in cultures of mDC and pDC with fixed or live CD3<sup>+</sup> T cells.**

Prior to co-culture, CD3<sup>+</sup> T cells from healthy donor peripheral blood (PB) were incubated in the presence of PHA (5μg/ml) for 48 hours. In half of experiments as denoted, T cells were then fixed in PBS/2%paraformaldehyde (Fixed) or left alone (Live). At 48 hours mDC and pDC were sequentially purified by magnetic cell sorting from the same donor. mDC and fixed (a) or live (b) T cells were cultured alone, or together at a ratio of 3:1(T cells:mDC) in the presence or absence of anti-TNF-α (100ng/ml), anti-IL-15 (100ng/ml) or mouse (m) IgG<sub>1</sub> (100ng/ml) negative control. In parallel assays, pDC and fixed (c) or live (d) T cells were cultured alone, or together at a ratio of 3:1(T cells:mDC) in the presence or absence of anti-TNF-α (100ng/ml), anti-IL-15 (100ng/ml) or mouse (m) IgG<sub>1</sub> (100ng/ml) negative control. CCL4 levels in culture supernatant were measured at 24 hours by multiplex analysis at 24 hours. Values are the mean ± SEM of triplicate cultures. Similar data was obtained from 6 healthy donors.

\*p<0.05

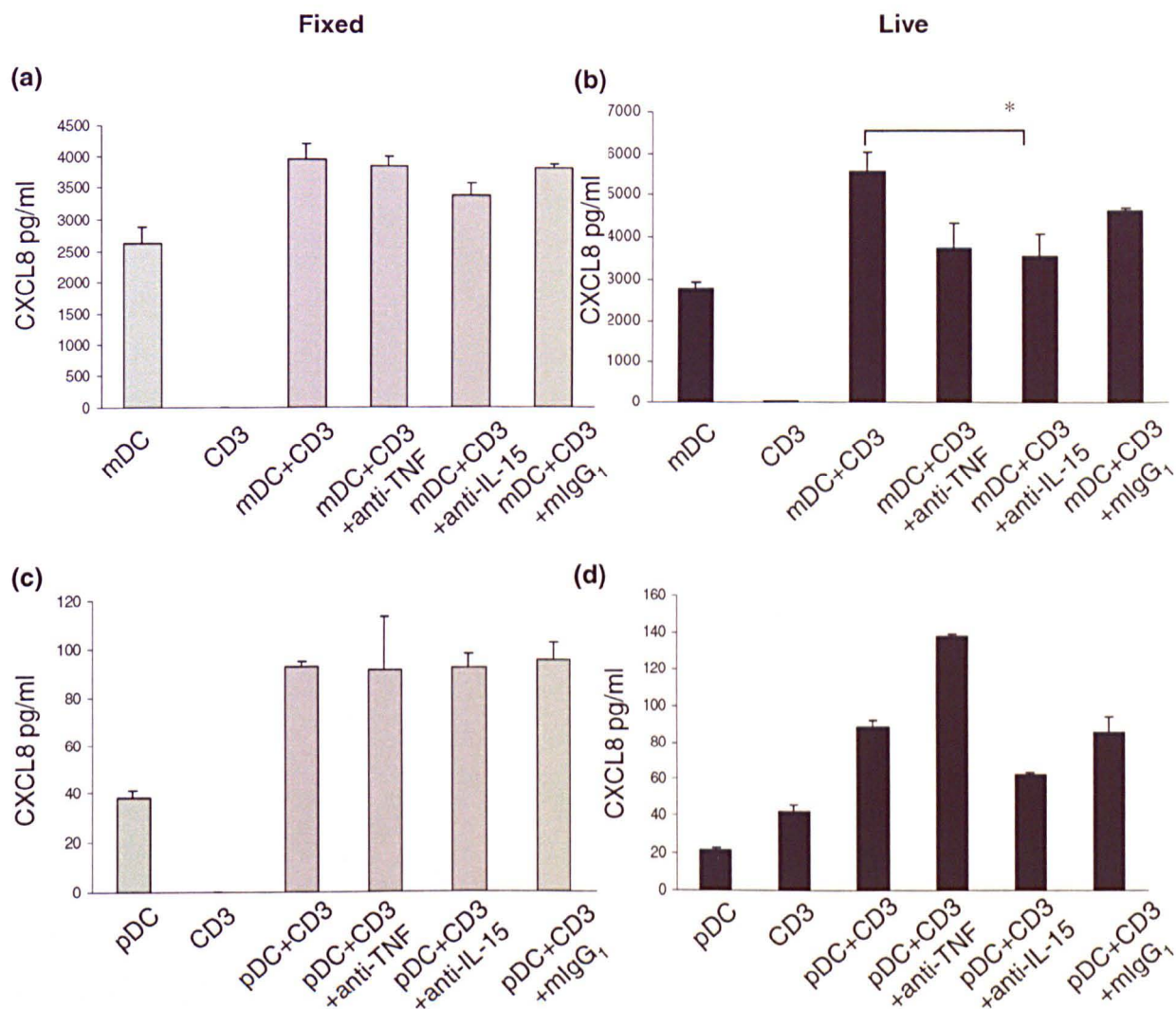


Anti-TNF $\alpha$  = 100ng/ml  
 Anti-IL-15 = 100ng/ml  
 Mouse IgG<sub>1</sub> = 100ng/ml

**Figure 5.17 CXCL8 production in cultures of mDC and pDC with fixed or live CD3<sup>+</sup> T cells.**

Prior to co-culture, CD3<sup>+</sup> T cells from healthy donor peripheral blood (PB) were incubated in the presence of PHA (5µg/ml) for 48 hours. In half of experiments as denoted, T cells were then fixed in PBS/2%paraformaldehyde (Fixed) or left alone (Live). At 48 hours mDC and pDC were sequentially purified by magnetic cell sorting from the same donor. mDC and fixed (a) or live (b) T cells were cultured alone, or together at a ratio of 3:1(T cells:mDC) in the presence or absence of anti-TNF-α (100ng/ml), anti-IL-15 (100ng/ml) or mouse (m) IgG<sub>1</sub> (100ng/ml) negative control. In parallel assays, pDC and fixed (c) or live (d) T cells were cultured alone, or together at a ratio of 3:1(T cells:mDC) in the presence or absence of anti-TNF-α (100ng/ml), anti-IL-15 (100ng/ml) or mouse (m) IgG<sub>1</sub> (100ng/ml) negative control. IL-8 levels in culture supernatant were measured at 24 hours by multiplex analysis at 24 hours. Values are the mean ± SEM of triplicate cultures. Similar data was obtained from 6 healthy donors.

\*p<0.05



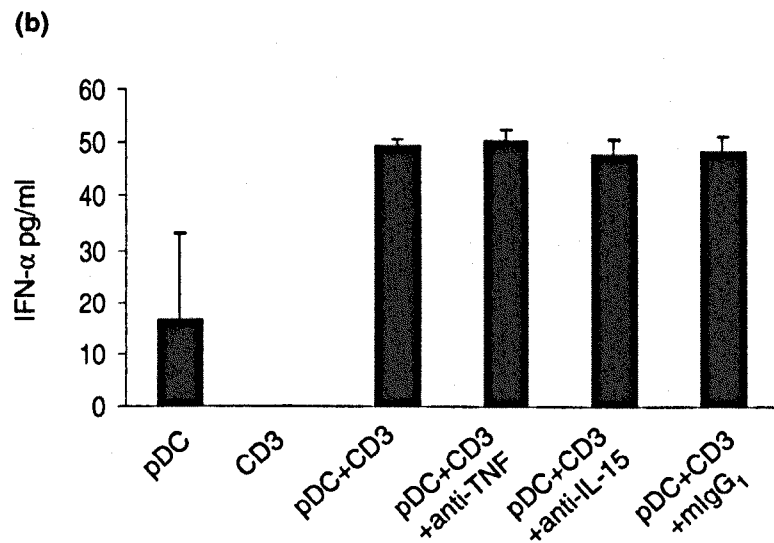
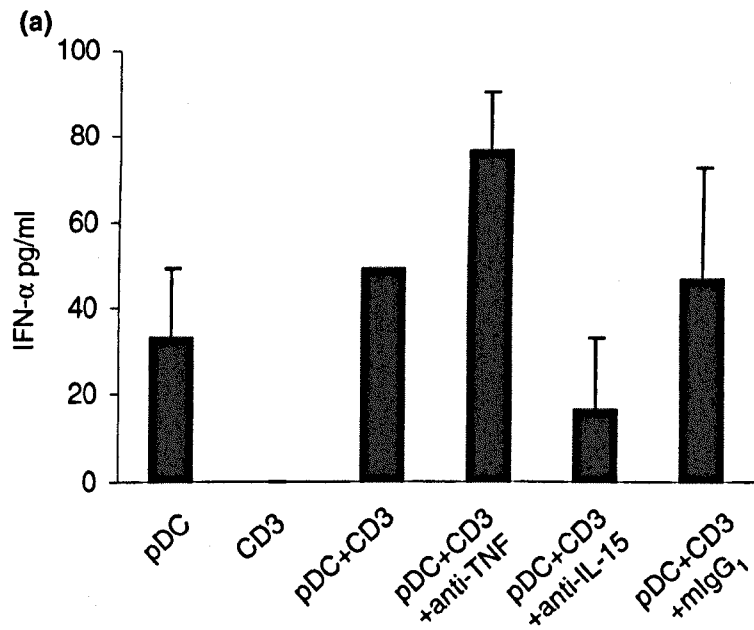
Anti-TNF $\alpha$  = 100ng/ml  
 Anti-IL-15 = 100ng/ml  
 Mouse IgG<sub>1</sub> = 100ng/ml

IFN producing cell in the body, we sought to evaluate the possibility that TNF- $\alpha$  neutralisation enabled IFN- $\alpha$  driven secretion of CCL3, CCL4 and CXCL8 from pDC. Multiplex analysis indicated that IFN- $\alpha$  was absent from mDC cultures, but was present at low levels in pDC cultures and enhanced by T cell contact (<100pg/ml). In one of the two donors from whom pDC produced chemokines were measurable, anti-TNF- $\alpha$  but not anti-IL-15, increased IFN- $\alpha$ , although this was not significant (Fig. 5.18a). In the second donor anti-TNF- $\alpha$  and anti-IL-15 did not alter IFN- $\alpha$  levels (Fig. 5.18b). Further analyses will be required in order to elucidate the role, if any, which TNF- $\alpha$  may have in the inhibition of IFN- $\alpha$  directed chemokine release from pDC.



**Figure 5.18 IFN- $\alpha$  production in cultures of pDC with live CD3<sup>+</sup>T cells.**

Prior to co-culture, CD3<sup>+</sup>T cells from healthy donor peripheral blood (PB) were incubated in the presence of PHA (5 $\mu$ g/ml) for 48 hours. At 48 hours pDC were sequentially purified by magnetic cell sorting from the same donor. pDC and live T cells were cultured alone, or together at a ratio of 3:1(T cells:pDC) in the presence or absence of anti-TNF- $\alpha$  (100ng/ml), anti-IL-15 (100ng/ml) or mouse (m) IgG<sub>1</sub> (100ng/ml) negative control. IFN- $\alpha$  levels in culture supernatant were measured at 24 hours by multiplex analysis at 24 hours. Values are the mean  $\pm$  SEM of triplicate cultures. The results from 2 healthy donors are shown (a) and (b).



Anti-TNF $\alpha$  = 100ng/ml  
Anti-IL-15 = 100ng/ml  
Mouse IgG<sub>1</sub> = 100ng/ml

## 5.4 Discussion

This chapter has addressed three key areas concerning cytokine modification of DC function and broader area of synovial inflammation. Firstly, we have examined DC subsets in synovium after TNF- $\alpha$  blockade. Second, we have examined the broader action of IL-15 on key DC derived cytokines within models of synovial inflammation. Third, and finally we have compared and contrasted the effects of both of these on DC subsets *in vitro*.

The advent of cytokine-targeted therapies, in particular TNF- $\alpha$  blockade, has revolutionised the treatment of RA and, increasingly, PsA. However, TNF- $\alpha$  blockade fails to control disease in around 50% of patients, so there remains considerable unmet clinical need. A critical goal now is to identify the exact mechanisms by which cytokine blockade acts, such that current treatment strategies can be optimised and potential synergistic targets identified. Our investigations thus far have suggested an active role for DC subsets in the perpetuation of chronic inflammation within the synovial compartment. To date, only one other group has attempted to assess the direct effect of cytokine blockade on DC subsets in arthritis in a human setting (680). However, that study analysed only peripheral blood DC subsets and did not attempt to examine the inflamed synovial compartment. This study has sought therefore to examine the effect of TNF- $\alpha$  neutralisation, particularly on DC subsets in RA and PsA synovium, and IL-15 neutralisation on DC subsets in order to address some of the outlying questions. Namely, what are the mechanisms of action of these therapies, and by elucidating these do we gain further insight into the contribution of DC to disease pathology?

We observed decreased mDC infiltration, but not pDC infiltration, to the synovium of RA and PsA patients 48 hours after a single infusion of infliximab (3 mg/kg). The decrease in mDC did not attain statistical significance, possibly attributable to their rarity in SM. However, our observation was consistent with previous analyses by the Division of Clinical Immunology and Rheumatology at the AMC, undertaken on the same tissue cohort, which demonstrated significant reduction in tissue infiltrating CD3<sup>+</sup> T cells and CD68<sup>+</sup> macrophages after infliximab infusion (668, 669). These studies found that the reduction in synovial cellularity could not be explained by TNF-blockade induced

apoptosis at the site of inflammation, which has been reported to occur in Crohn's disease (681, 682).

It is important to note that these findings are in contrast to a recent report by Catrina et al. linking infliximab induced decrease of synovial tissue CD3<sup>+</sup> T cells and CD68<sup>+</sup> macrophages to apoptosis, in particular of macrophages (683). However, this discrepancy might be explained by a number of factors. First, in contrast to our assessment of the inflammatory infiltrate after 48 hours, the latter report analysed SM infiltrate 8 weeks after infliximab infusion. It is possible that the apoptotic events described by Catrina et al. occurred secondary to infliximab infusion, as a later event of inflammatory amelioration rather than an early mechanism of cellular reduction. Second, the specific link to macrophage apoptosis was identified by *in vitro* Annexin V analysis of synovial fluid (SF) derived macrophages. Annexin V binds phosphatidylserine (PS), externalised to the cellular surface early in apoptosis. However, PS externalisation is not exclusive to apoptosis, but can be triggered by intracellular calcium mobilisation and its expression on the surface of non-apoptotic primary macrophages is a requirement for the engulfment of apoptotic target cells (684, 685). As a result, Annexin V staining is not a good method by which to assess macrophage apoptosis. Furthermore, Catrina et al. did not find any evidence of infliximab induced SF CD3<sup>+</sup> T cell apoptosis, and as such apoptosis fails to explain their rapid efflux from the synovium.

We cannot exclude the possibility that infliximab induced apoptosis in DC subsets in compartments other than the SM, such as the PB, thereby affecting migration towards the synovial compartment. However, this possibility is unlikely. First, a study by Balanescu et al., examining DC subsets in RA PB pre- and post-infliximab infusion detected no significant decrease in CD11c<sup>+</sup> mDC or CD123<sup>+</sup> pDC, although surface CD83 was down-regulated on both subsets (535). It is of course possible in that study that the detection of mDC and pDC using CD11c and CD123 respectively, which are not specific to either subset (discussed in chapter 3), may have resulted in failure to detect comparatively small alterations in these rare cell types. Second, the aforementioned study by Catrina et al., detected no significant death in infliximab treated RA PBMC derived CD3<sup>+</sup> T cells or CD14<sup>+</sup> monocytes (683).

Instead, we propose that infliximab infusion altered local SM adhesion molecule and chemokine expression, reducing leukocyte ingress to the SM, as has been demonstrated in other studies (662, 663). Our own observation that TNF- $\alpha$  neutralisation down-regulates T cell cognate induced mDC secretion of the inflammatory chemokines CCL3, CCL4 and CXCL8, is consistent with such a hypothesis. CCL3, CCL4 and CXCL8 are particularly chemotactic for monocytes, effector T cells and neutrophils and their release by immature DC upon TLR stimulation or CD40 ligation is considered a crucial first step in the initiation of an inflammatory response (686). However, DC release of these chemokines is rapidly downregulated as DC mature. As we have demonstrated (chapter 4), the arthritic SM contains large numbers of immature DC whose maturation may be inhibited by local TNF- $\alpha$ . In the absence of maturation it is possible that DC inflammatory chemokine release is not down-regulated, contributing to the continued recruitment of leukocytes to the SM. In addition to triggering mDC efflux from the SM, TNF-blockade could also enable DC maturation and down-regulation of CCL3, CCL4 and CXCL8, resulting in reduced immigration of leukocytes to the SM.

In contrast to mDC, we made the intriguing observation that pDC in RA SM were, if anything increased 48 hours after infliximab infusion. In PsA however, pDC were slightly decreased in SM 48 hours after infliximab infusion. Given the low number of PsA patients examined in this study it is impossible to draw any conclusions about potential differences between diagnostic groups. Furthermore, neither of the observed alterations achieved statistical significance potentially due to cell rarity, low number of participant patients or the early time point examined. Nevertheless, the study may indicate trends suggesting the fascinating possibility that TNF-blockade affects DC subsets differentially. Such a supposition is consistent with our *in vitro* analyses that indicated TNF- $\alpha$  neutralisation induced an *increase* of the pro-inflammatory chemokines CCL3, CCL4 and CXCL8 from pDC co-culture with T cells. A number of groups have suggested that TNF- $\alpha$  inhibits the action and secretion of IFN- $\alpha$  (591) and IFN- $\alpha$  has been demonstrated to drive CCL2, CCL3 and CCL4 secretion from pDC, but not mDC (679). Conceivably, TNF- $\alpha$  neutralisation could enable the action of IFN- $\alpha$ . Consistent with this hypothesis, we detected low levels of IFN- $\alpha$  in pDC culture supernatants, but not mDC culture supernatants. Furthermore, IL-15 neutralisation did not increase pro-inflammatory chemokines in pDC culture – suggesting a TNF- $\alpha$  specific affect. Future analyses of the

interplay of IFN- $\alpha$  and TNF- $\alpha$  on pDC would be required in order to explore this hypothesis further. Elucidating such a mechanism may be of particular import given the high level of IFN- $\alpha$  expressing pDC in SM (chapter 4) and reports linking IFN- $\alpha$  infusion to arthritis induction (545, 619). Potentially, this could offer an explanation as to why some patients fail TNF- $\alpha$  therapy, while others do not. It would be of particular interest to examine IFN- $\alpha$  levels in SM pre- and post-TNF blockade, in order to evaluate any correlation to disease response. Since 90% of patients responded to infliximab therapy in this study, we were unable to ascertain any significant information on the potential correlation of DC subsets in SM to patient response.

A further unexpected, but potentially informative observation from this study was the low overall pDC infiltration to the RA and PsA SM at baseline. This was in contrast to our earlier examinations identifying relatively large numbers of pDC, compared to mDC, in both RA and PsA SM (chapter 4). Importantly, all patients in the infliximab study were on a stable, high dose course of methotrexate (up to 25 mg/kg). It is possible that the low pDC infiltrate was directly incurred by methotrexate treatment. Alternatively, it may reflect a lower DAS28 in this patient cohort than in those in the earlier studies. Of interest, mDC in SM at baseline did not appear altered as compared to our earlier studies, suggesting the intriguing possibility that methotrexate affects DC subsets differentially, but unlike TNF- $\alpha$  blockade, in a pDC specific manner. Of note, adenosine, a metabolic factor of methotrexate, has been demonstrated to be chemotactic for immature pDC, but inhibits CpG ODN stimulated cytokine release from pDC (687). This may also identify a mechanism by which methotrexate complements TNF-blockade in synergistic therapeutic treatment. The possibility that the reduced pDC count occurred due to the technical difficulties inherent in single session processing of such a large number of samples cannot be discounted. In either case, further *ex vivo* analysis is necessary and would be informative.

An important observation of the current study was the different biological mechanisms of action of IL-15 neutralisation in contrast to TNF- $\alpha$  neutralisation. We first demonstrated that IL-15 neutralisation inhibited IFN- $\gamma$  release from SF mononuclear cells cultured in the presence of exogenous IL-12 and IL-15. We next found evidence that IL-15 neutralisation also reduced spontaneous TNF- $\alpha$  release from SF mononuclear cells, consistent with

neutralisation of endogenous IL-15. Together these data support an upstream role for anti-IL-15 mediated inhibition of pro-inflammatory cytokines.

Other factors are also likely to be significant biological targets of IL-15 neutralisation. This laboratory has previously described the anti-IL-15 mediated reduction of TNF- $\alpha$  release by macrophages induced by activated T cell contact (631). Here, for the first time, we show that anti-IL-15 also significantly reduces TNF- $\alpha$  released by mDC-T cell and pDC-T cell contact dependent cultures. Furthermore, CCL3 and CXCL8 were significantly reduced in mDC-T cell cultures, while the trend was towards a reduction in CCL3, CCL4 and CXCL8 in all mDC-T cell and pDC-T cell contact dependent cultures. The fact that TNF- $\alpha$  and the pro-inflammatory chemokines were only reduced in live, but not fixed, T cell cultures was particularly informative. Firstly, we have demonstrated that IL-15 antibody induces CD69 down-regulation of surface CD69. CD69 is both a marker of T cell activation and a molecule implicated in T cell-APC interactions at a functional level (631) thus its down-regulation is likely to hinder DC-T cell contact. However, this is an active process that cannot occur after T cell fixation thus CD69 down-regulation is implicated in the efficacy of anti-IL-15 in live T cell cultures. Secondly, as demonstrated here, IL-15 neutralising antibody can bind membrane bound IL-15. DC membrane bound IL-15 tightly co-regulates IL-2 production, a requirement of DC directed T cell activation, which in turn enhances release of pro-inflammatory cytokines from DC. Again, this mechanism is inhibited by T cell fixation. The direct neutralisation of IL-15 at this critical point in live cell contact therefore significantly impaired this process. Finally, and of particular note, IL-15 has recently been shown to have a critical role in the cross-regulation between DC subsets related to autocrine IL-15 enhanced CD40 expression (688). Therefore down-regulation of CD40 expression offers a third critical point at which cognate T cell-DC interactions can be blocked by IL-15 neutralisation. Moreover, it suggests a mechanism by which pDC-mDC contact could also be disrupted. Since the majority of mDC and pDC in SM expressed IL-15 and both subsets were localised to T cell aggregates (chapter 4), this cognate interaction constitutes an important target of IL-15 therapy by limiting DC driven T cell expansion and DC proinflammatory cytokine and chemokine release.

The search for new approaches through which disease amelioration can be achieved continues, and while promising candidates such as CTLA-4 Ig and CD20 have been developed, they have currently proved no more efficacious than cytokine blockade. The foregoing data indicates that TNF- $\alpha$  and IL-15 blockade may have biologically distinct, yet complementary mechanisms of action, in particular in regards their affects on DC subsets in T cell contact. As such, IL-15 targeting may, like methotrexate, be a potential candidate for synergistic therapeutic action alongside TNF- $\alpha$  blockade. Furthermore, these studies have indicated that the contribution of mDC and pDC to disease pathology may be different, as exemplified by the differential affects of TNF-blockade and possibly, methotrexate treatment. This is consistent with our earlier observations that mDC and pDC in the inflamed SM express distinct cytokine profiles. However, it is difficult to unravel the relative contribution of mDC and pDC to disease initiation and perpetuation in the absence of experimental models, since one particular impediment to the functional analysis of immune cells within human disease is the lack of access to the joint proximal lymph node – a critical compartment particularly for the induction of autoimmune T cells. An important pre-requisite to the inference of particular immune pathways and cell types to disease pathology is therefore specific analysis within an *in vivo* disease setting. Thus in order to further elucidate the diverse functions of pDC and mDC in inflammatory arthritis it was necessary to investigate their action in a murine model of arthritis. The results of these analyses will be discussed in chapter six.



## **Chapter 6: Analysis of the functional role of pDC in a murine model of arthritis**

## 6.1 Introduction

The development of central tolerance in the thymus, whereby self-reactive T cells are eliminated through clonal deletion is an imperfect process, allowing some self-reactive lymphocytes to escape to the extra-thymic regions. Self-reactive lymphocytes are consequently present in the normal immune repertoire (500). The maintenance of peripheral tolerance by dendritic cells (DC), through cell-contact and cytokine mediated modulation of the immune response, is therefore critical in preventing pathological self-reactivity. However, genetic and/or environmental factors can impair DC function and peripheral tolerance can fail, resulting in autoimmune diseases such as rheumatoid arthritis (RA) and psoriatic arthritis (PsA). While human studies, particularly those thus far described, have indicated a clear role for DC in the perpetuation of chronic inflammation, their role in disease initiation, in particular as it pertains to breach of tolerance has been impaired.

Murine models have been fundamental in elucidating the specific mechanisms by which DC may, or may not, induct disease *in vivo*. In particular, numerous animal models of inflammatory arthritis have been instrumental in elucidating the aetiology and pathogenetic mechanisms of articular inflammation. In particular, the early infiltration of (m) DC to the target tissue of inflammation in collagen-induced arthritis (CIA), spontaneous arthritis in MRL/*lpr* mice (550) and antigen-induced arthritis in rats (551, 552) has been demonstrated. Brewer and Leung demonstrated the capacity for DC to induce arthritis using *ex vivo* collagen type II-pulsed, bone-marrow differentiated mDC, to induce inflammation in joints adjacent to the site of injection (553). Furthermore, administration of TNF antagonists was sufficient to inhibit arthritic progression in this model. Similarly, adoptive transfer of T-box expressed in T cells (T-bet) expressing DC was sufficient to re-constitute inflammation in RAG2<sup>-/-</sup>Tbet<sup>-/-</sup> mice, otherwise resistant to collagen antibody-induced arthritis (554). Of particular interest, transfer of DC, genetically modified to over-express IL-4, to murine with established CIA reduced IFN- $\gamma$  production and collagen type II reactivity thus reducing disease severity and demonstrating a potential for manipulation of DC as a therapeutic vector (555). To date, no comparative analysis of the functional contribution of distinct DC subsets, in particular pDC, to murine models of arthritis has been performed.

In the mouse, up to six DC subtypes have been identified and can be distinguished by CD4 and CD8 expression. Three distinct myeloid CD11c<sup>hi</sup> DC subtypes have been delineated in spleen, CD4<sup>-</sup>/CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>-</sup>/CD8<sup>-</sup>. Lymph nodes contain two extra subtypes thought to have migrated from the tissue to the lymph nodes, CD4<sup>-</sup>/CD8<sup>-</sup>/CD205<sup>lo</sup>/CD11b<sup>+</sup> interstitial DC and CD4<sup>-</sup>/CD8<sup>lo</sup>/CD205<sup>hi</sup>/langerin<sup>+</sup> langerhans cells (416-418). In recent years a CD4<sup>-/+</sup>/CD8<sup>-/+</sup> plasmacytoid DC subset has been identified and can be distinguished from myeloid DC by CD11c<sup>lo</sup>/MHC II<sup>lo</sup>/B220<sup>hi</sup> /120G8<sup>+</sup> /mPDCA-1<sup>+</sup> expression (419-422). Murine DC subsets are remarkably phenotypically similar to their human counterparts, with the exception of CD8 expression, although no evidence suggests this is functionally significant. In addition murine, but not human, pDC express CD11c, albeit at low levels.

## 6.2 Aims and Objectives

The studies examining mDC and pDC in RA and PsA thus far described, indicate a pathological role for both DC subsets, in particular as regards altered migration to the inflamed synovial compartment and lymphocyte aggregates therein, and expression of pro-inflammatory cytokines that may expand Th17 cells and perpetuate chronic inflammation. The aim of this chapter was to undertake an *in vivo* analysis of DC subsets within a murine model of arthritis in order to elucidate their comparative roles in disease induction.

Specifically, I planned to address:

- Whether mDC and pDC may (also) migrate towards to the lymph node, as well as the target inflamed synovial tissue, during a murine model of chronic arthritis. Specifically it was planned to examine draining lymph nodes from DBA/1 undergoing collagen-induced arthritis.
- To determine if a culture system could be developed by which relatively large numbers of murine bone marrow (BM) derived pDC could be differentiated and processed to high purity, for future *in vitro* functional analysis and potential adoptive transfer experiments.
- To compare the ability of murine BM differentiated mDC and pDC to process and present antigen, by specific examination of their respective abilities to induce memory T cell proliferation to OVA and collagen type II.

- Finally, to deplete pDC using pDC specific depleting antibody 120G8 during the OVA TcR transgenic T cell adoptive transfer model of murine arthritis in order to gain insight into the functional role of pDC and, by association, mDC to the pathogenesis of this model.

## 6.3 Results

### 6.3.1 Animals

6-8 week old female BALB/c mice were used for *in vitro* experiments and as Tg CD4<sup>+</sup>/KJ1.26<sup>+</sup> T cell transfer recipients in the OVA TcR transgenic model of arthritis. DO11.10 BALB/c TCR Tg mice containing CD4<sup>+</sup>T cells expressing a TCR that recognizes the chicken OVA peptide 323-339 complexed with the MHC class II molecule I-Ad (detected by the clonotypic mAb KJ1.26) (574), were used as Tg CD4 T cell donors (bred in-house, Central Research Facility, University of Glasgow, Glasgow, UK). 6-8 week old female DBA/1 mice were purchased from Harlan (Bicester, UK) and used for *in vitro* experiments and in the collagen-induced arthritis (CIA) model.

### 6.3.2 pDC are expanded in the draining lymph nodes during collagen-induced arthritis.

In RA and PsA, as we demonstrated in chapters 3 and 4, DC subsets are significantly reduced in the peripheral blood (PB) circulation; this may be explained by their preferential accumulation to the inflamed synovial compartment. Since reduced PB DC numbers could be correlated to active inflammation (as determined by CRP levels) and DC are known to migrate rapidly to the lymph nodes (LN) following inflammatory stimulation (689), it is likely that the reduction could reflect at least in part LN directed migration. However, due to ethical and technical restrictions we were unable to investigate if mDC and/or pDC also accumulated in the human LN. We therefore chose to investigate this possibility by analyzing DC subsets in the draining LN of DBA/1 mice undergoing collagen-induced arthritis (CIA), an extensively characterized model of chronic arthritis.

Draining popliteal and inguinal LN were harvested from DBA/1 mice undergoing CIA (24-25 days into model) and naïve DBA/1 mice. DC subsets were detected in single-cell suspensions by CD11c<sup>high</sup> (mDC) and mouse plasmacytoid dendritic cell antigen (mPDCA)-1<sup>+</sup> (pDC) expression, followed by fluorescent-activated cell sorting (FACS) analysis of viable cells. In naïve DBA/1, pDC were the dominant DC population in the LN, comprising ~0.36% of all LN cells compared to ~0.29% for mDC (Fig. 6.1a and c, respectively). In CIA DBA/1, pDC were expanded in the draining LN to ~0.8% of all cells

(Fig. 6.1b). In contrast, mDC comprised ~0.6% of all cells however this did not represent subset expansion when compared to the matched isotype control (Fig. 6.1d). The data indicate that pDC in the LN are expanded two-fold during chronic articular inflammation, commensurate with the LN as a target for pDC migration.

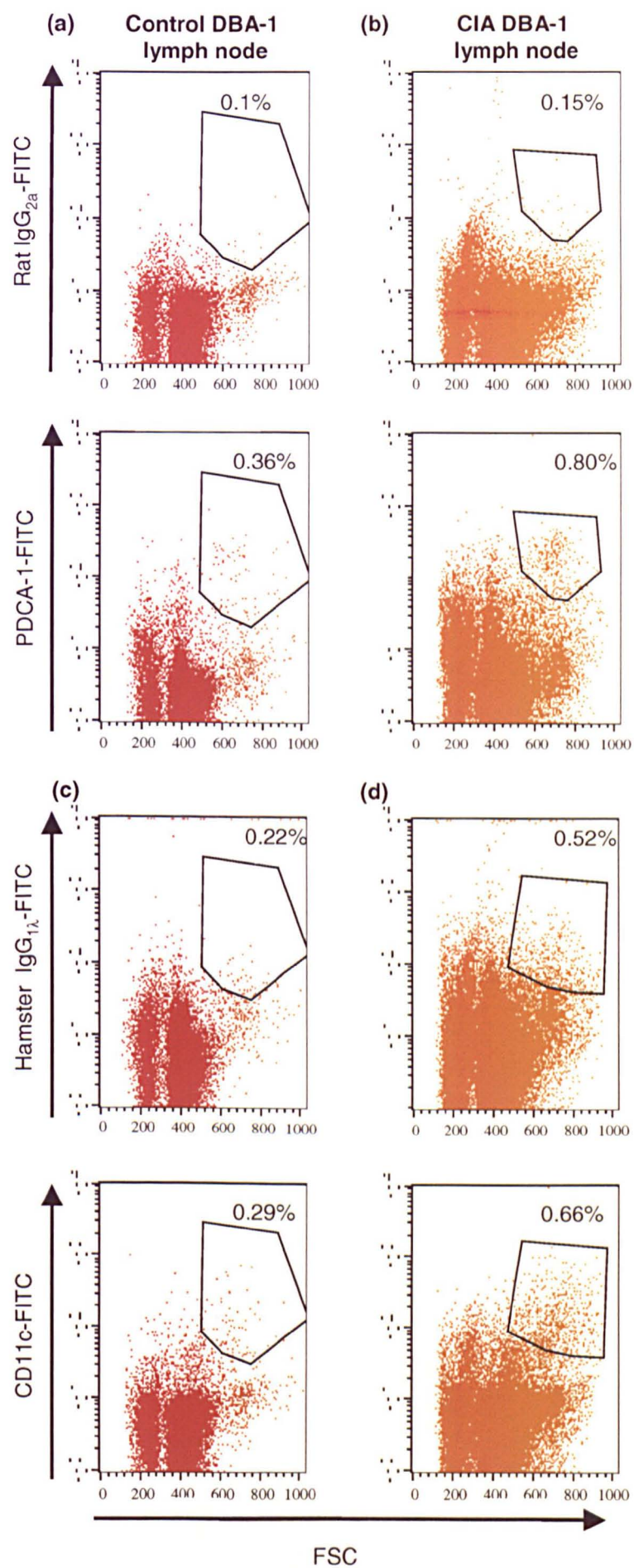
### 6.3.3 Culture of pDC from murine bone marrow

Previous studies from this laboratory have demonstrated that administration of collagen-pulsed bone-marrow derived mDC to DBA/1 recipients is sufficient for the induction of arthritis (553). There have to date been no similar analyses of pDC in models of chronic arthritis, attributable to the technical challenges encountered when working with this rare cell population. It has been established that pDC do not phagocytose, process and load antigens onto MHC molecules as efficiently as mDC, although they can promote proliferation in antigen experienced T cells (453, 690). However, it is not clear whether pDC are able to directly prime naïve T cells. In addition, pDC surface MHC II and co-stimulatory molecule expression is lower than mDC (422). Thus the competence of pDC antigen-presentation is currently a controversial issue, and it has been hypothesized that they act complementary to mDC, inducing the differentiation of unpolarized T cells previously expanded by mDC (453, 690). We therefore considered it necessary to investigate the ability of pDC to process and present antigen by a panel of *in vitro* assays, prior to any potential analysis of their *in vivo* influence on arthritis. To do this it was first necessary to develop a method by which pDC could be differentiated from murine bone marrow.

The differentiation of mDC from murine bone marrow by supplementation using GM-CSF is well-established in the literature. The differentiation of pDC from murine bone marrow, however, is not well characterized - at best 70%pDC in culture can be achieved (691, 692). Culture of murine bone marrow in RPMI 1640 complete medium/10%FCS supplemented with human Flt-3L (100ng/ml) gave a peak pDC population of routinely ~60% purity by day 7 of culture, as determined by mPDCA-1<sup>+</sup> expression (Fig. 6.2a). Around 80% of cells were CD11c<sup>+</sup> (Fig. 6.2b) but isolated murine pDC are CD11c<sup>lo</sup> and those generated from bone marrow have been demonstrated to stain high for CD11c (691) thus only 25% of these were likely to be APC other than pDC. Cultures were then purified further by mPDCA-1 positive magnetic cell sorting and application to two magnetic columns. While this method yielded a relatively pure population, routinely >95%, as assessed by

**Figure 6.1 pDC, but not mDC are expanded in the draining lymph nodes in collagen-induced arthritis.**

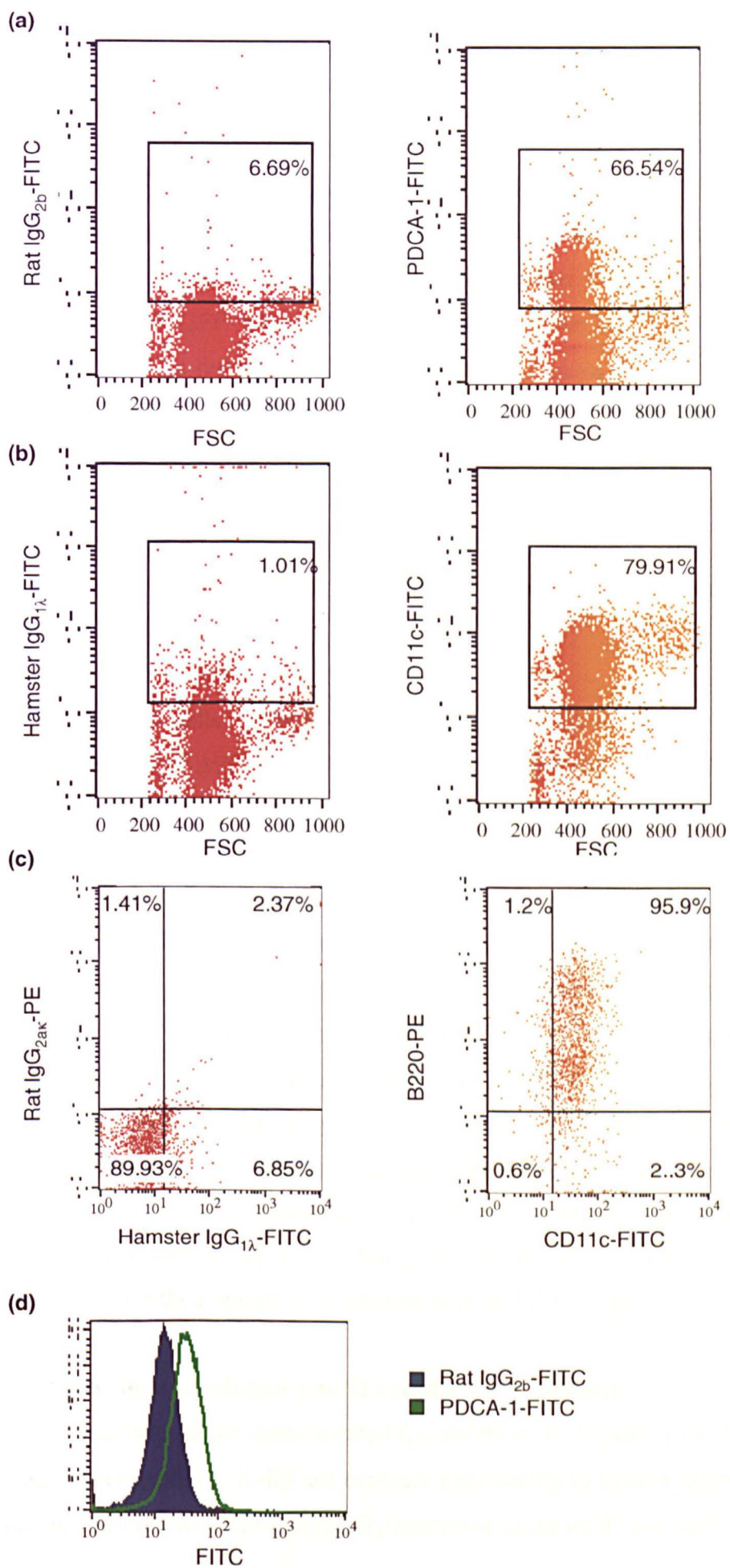
The number of mDC and pDC in lymph nodes taken from normal DBA-1 mice or DBA-1 mice undergoing collagen induced arthritis was assessed by staining with PDCA-1 (pDC) in comparison to the isotype control (**a** and **b**) or CD11c (mDC<sup>high</sup>/pDC<sup>lo</sup>) in comparison to the isotype control (**c** and **d**) and analysed by FACS.





**Figure 6.2 Phenotype of rhFlt-3L cultured murine bone marrow cells.**

The percentage of pDC in murine bone marrow cells cultured with 100ng/ml rhFlt-3L was determined on day 7 of culture by staining with mPDCA-1 (pDC) compared to isotype control Rat IgG<sub>2a</sub> (a) or CD11c (mDC<sup>high</sup>/pDC<sup>lo</sup>) compared to isotype control Hamster IgG<sub>1 $\lambda$</sub>  (b) and analysing by FACS. The purity of pDC after positive selection using mPDCA-1 microbeads was determined by double staining with B220/CD11c as compared to the isotype control (c) and confirmed using PDCA-1 (d).



CD11c<sup>+</sup>/B220<sup>+</sup> staining (Fig. 6.2c) and confirmed by mPDCA-1<sup>+</sup> staining (Fig. 6.2d), the actual cell yield was very low.

#### **6.3.4 mDC and pDC present OVA peptide to DO11.10 GFP transgenic T cells.**

We first examined the antigen-presentation potential of BALB/c bone marrow differentiated mDC and pDC by presentation of the chicken OVA peptide 323-339 (OVA<sub>323-339</sub>) to DO11.10 GFP transgenic (Tg) T cells. The DO11.10 GFP T cell hybridoma is a cell line that responds to presentation of OVA<sub>323-339</sub> on the MHC Class II molecule (I-A<sup>d</sup>). When the T cell hybridoma receives appropriate stimulation, it induces transcription of Nuclear Factor of activation of T cells (NFAT). Modification of the hybridoma by the addition of a NFAT reporter construct means that when NFAT is produced, it binds to a NFAT specific binding site, which regulates a green fluorescent protein (GFP) inducing promoter (575). In essence, when the T cell hybridoma sees MHC/peptide complex, it fluoresces.

Culture of DO11.10 Tg T cells for 24 hours, with or without OVA, did not induce GFP production in the absence of APC (Fig. 6.3a and b). Similarly, culture of DO11.10 Tg T cells for 24 hours with bone marrow derived mDC, but no OVA, did not induce T cell fluorescence (Fig. 6.3c). However, co-culture of DO11.10 Tg T cells with bone marrow derived mDC pre-pulsed with OVA for 6 hours, induced GFP production (Fig. 6.3d) illustrating the necessity for both MHC and peptide to induce transcription of NFAT. In parallel assays, DO11.10 Tg T cells were co-cultured with bone marrow derived pDC alone (Fig. 6.3e), or pDC that had been pre-pulsed with OVA (Fig. 6.3f). As with mDC, pDC pre-pulsed in OVA induced fluorescence in the DO11.10 Tg T cells. The data indicate therefore, that pDC are competent at antigen uptake, processing and presentation by MHC/peptide complex. In order to enhance the surface expression of MHC II and co-stimulatory receptors, pDC were stimulated with the TLR9 specific CpG ODN 1826 2 hours prior to co-culture with the Tg T cells, but after OVA pulsing. However, this did not appear to further enhance their antigen-presentation potential (Fig. 6.3g).

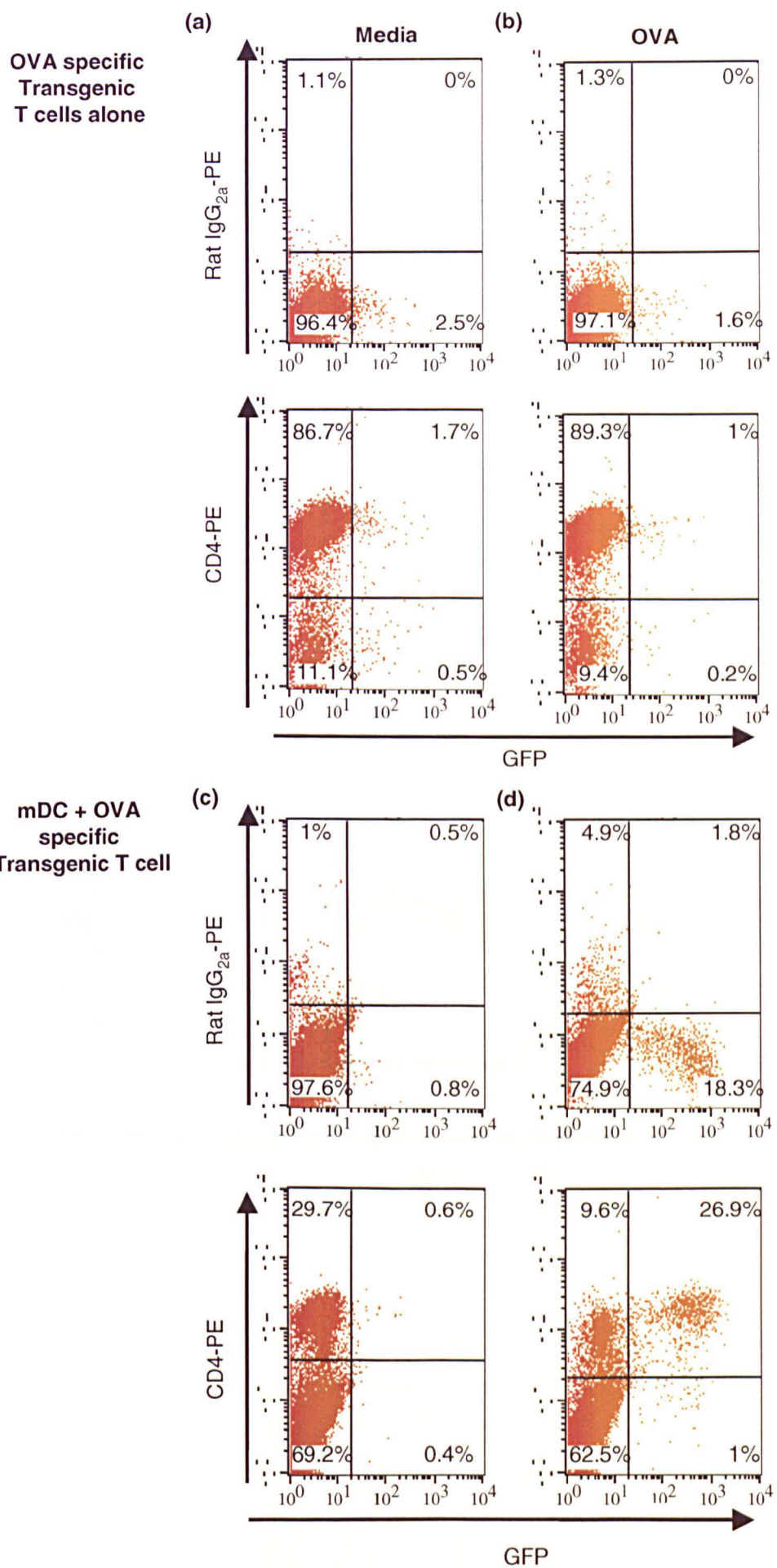
#### **6.3.5 mDC and pDC induce collagen type II specific T cell expansion.**

While the above described model demonstrated the ability of both mDC and pDC to take up, process and present antigen, it did not evaluate their ability to induce proliferation of the target cell. In addition, we were specifically interested in the mDC and pDC uptake of

**Figure 6.3 mDC and pDC present OVA peptide to D011.10 GFP transgenic T cells.**

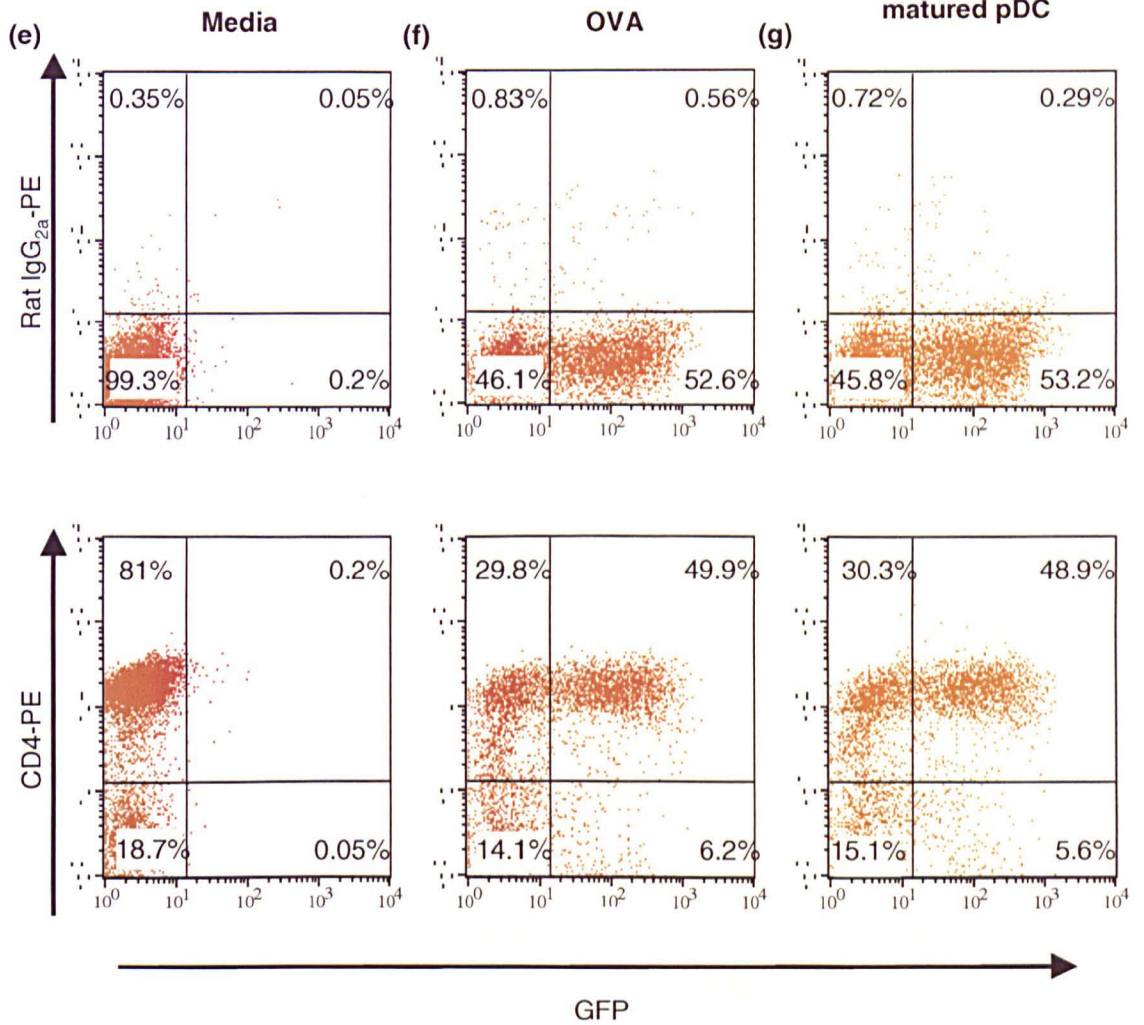
FACS analysis of CD4 expression (PE, FL2 channel) and GFP fluorescence (FL1 channel) on OVA transgenic D011.10 GFP T cells incubated for 24 hours alone (a), with 5mg/ml OVA (b), with mDC (c), mDC pre-pulsed for 6 hours with 5mg/ml OVA (d), pDC (e), pDC pre-pulsed for 6h with 5mg/ml OVA (f) or pDC pre-pulsed for 6h with 5mg/ml OVA followed by 2h with 3.2µg/ml CpG ODN 1826 (g). OVA transgenic D011.10 cells are identified as CD4<sup>+</sup> and when engaging OVA peptide on MHC II molecules fluoresce green thus becoming double positive CD4<sup>+</sup>/GFP<sup>+</sup>.

One example of three replicable experiments is shown.



pDC + OVA specific  
transgenic T cell

OVA +  
CpG ODN 1826  
matured pDC



Type II collagen (CII). CII is the most abundant fibrillar protein of articular cartilage and an important autoantigen in the pathogenesis of CIA in mice and RA in humans (296, 693). It has previously been demonstrated that mDC predominantly take CII up by macropinocytosis (694) a function at which pDC are generally considered poor (449). We therefore chose to examine the ability of bone marrow derived pDC and mDC to present collagen to CD4<sup>+</sup> T cells, purified by positive magnetic cell sorting from the draining lymph nodes of CIA DBA-1 mice, in comparison to naïve animals. Since CII alone has been shown to deliver a maturation stimulus to DC, possibly via TLR4, additional inflammatory stimuli such as LPS were not considered necessary to enable DC induced T cell proliferation (694, 695). To ensure purification of sufficient numbers of CD4<sup>+</sup> T cells from naïve mice, both lymph nodes and spleen were harvested. Although APC in the spleen also express CD4, the contamination of these after purification was <3% as assessed by CD11c<sup>+</sup> staining (Fig. 6.4a).

CII (50µg/ml) pulsed mDC induced a low level of proliferation in CD4<sup>+</sup> T cells from naïve DBA/1 mice as measured by thymidine incorporation after 72 hours co-culture (Index of Stimulation, 1.73, p=0.049) (Fig. 6.4b). In contrast, CII (50µg/ml) pulsed pDC did not induce any significant proliferation in CD4<sup>+</sup> T cells (Index of Stimulation, 1.15) (Fig. 6.4c). Arguably, an index of stimulation less than 2 may not indicate 'real' proliferation but reflect culture artefact. Alternatively, the low level proliferation induced in mDC/T cell cultures may be indicative of a small number of self-reactive T cells in culture and /or suboptimal timepoint of evaluation. I-A<sup>q</sup> haplotype possessing DBA/1 mice are highly susceptible to CIA and it has been shown that expansion of antigen-specific CD4<sup>+</sup> T cells after intradermal injection of CII emulsified in complete Freund's adjuvant (CFA) occurs early *in vivo*, before the onset of arthritis (696). We have also observed that during CIA, some animals develop arthritis (as assessed by paw swelling) prior to day 21 CII challenge (unpublished observations). Potentially these data may suggest a role for mDC, but not pDC in the initial CD4<sup>+</sup> T cell expansion phase of CIA.

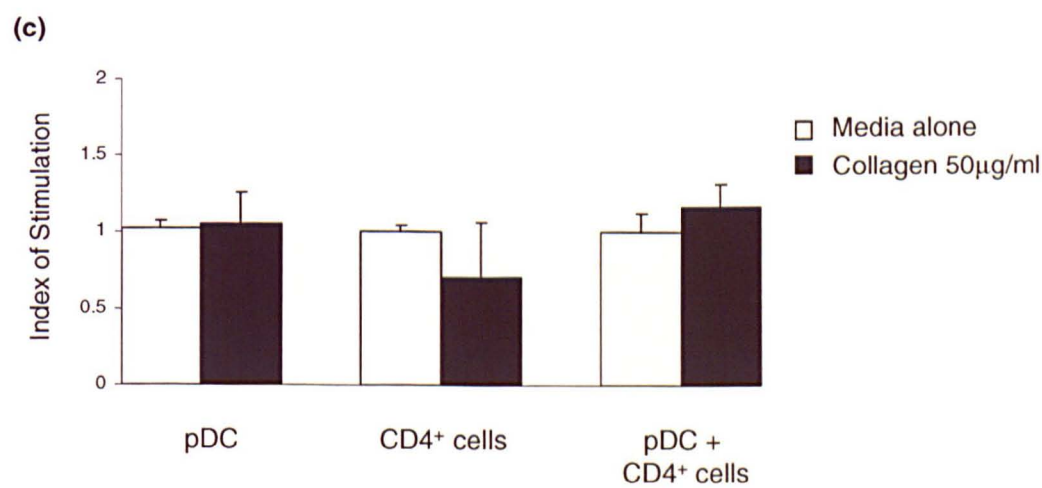
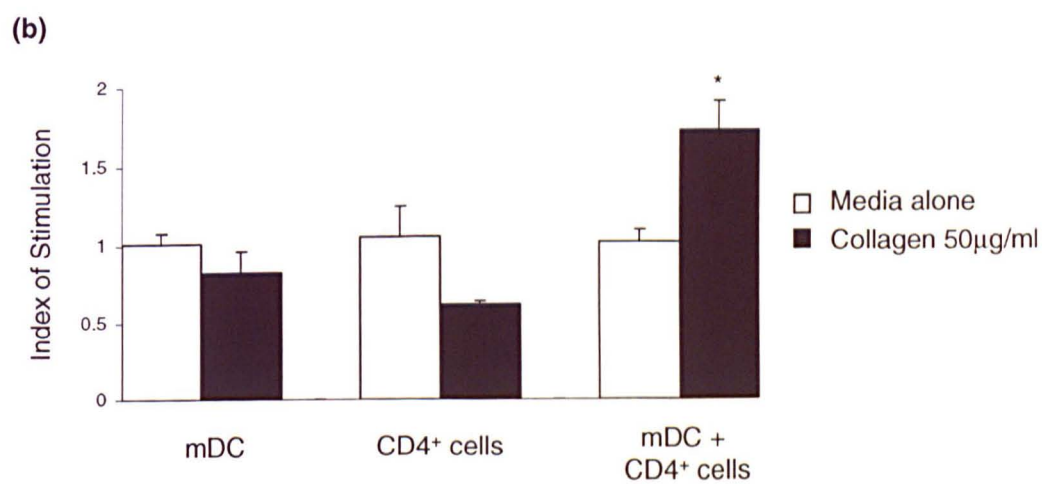
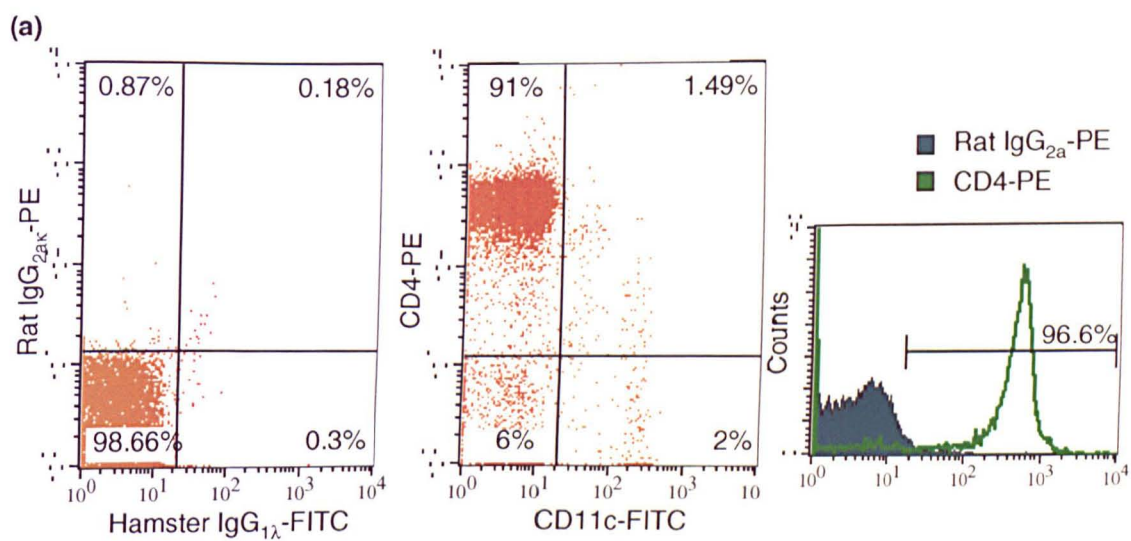
Purity of CD4<sup>+</sup> T cells isolated from the draining popliteal LN from DBA/1 mice undergoing CIA was routinely >96% (Fig. 6.5a). CII (50µg/ml) pulsed mDC induced a significant high level of proliferation in CD4<sup>+</sup> T cells from CIA DBA-1 mice as measured by thymidine incorporation after 72 hour co-culture (Index of Stimulation, 3.524, p=0.001)

**Figure 6.4 Collagen pulsed mDC, but not pDC, induce proliferation in naïve CD4<sup>+</sup> T cells.**

The phenotype of CD4<sup>+</sup> T cells purified from spleen and lymph nodes taken from naïve DBA-1 mice was confirmed by FACS (**a**, **b** and **c**) and revealed less than 3.5% contamination with CD11c<sup>+</sup> cells. The proliferation of the CD4<sup>+</sup> T cells following 72h incubation with either bone marrow derived mDC in media alone or containing 50µg/ml collagen (**d**) or bone marrow derived pDC in media alone or containing 50µg/ml collagen (**e**) was assessed by thymidine incorporation. Results are expressed as the stimulation index of an experiment carried out in triplicate. Results are representative of three replicable experiments.

\*<0.05

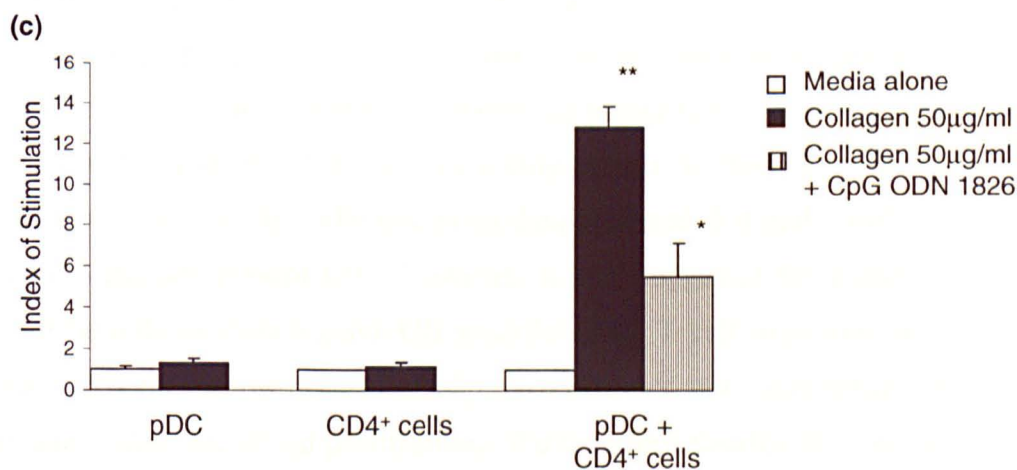
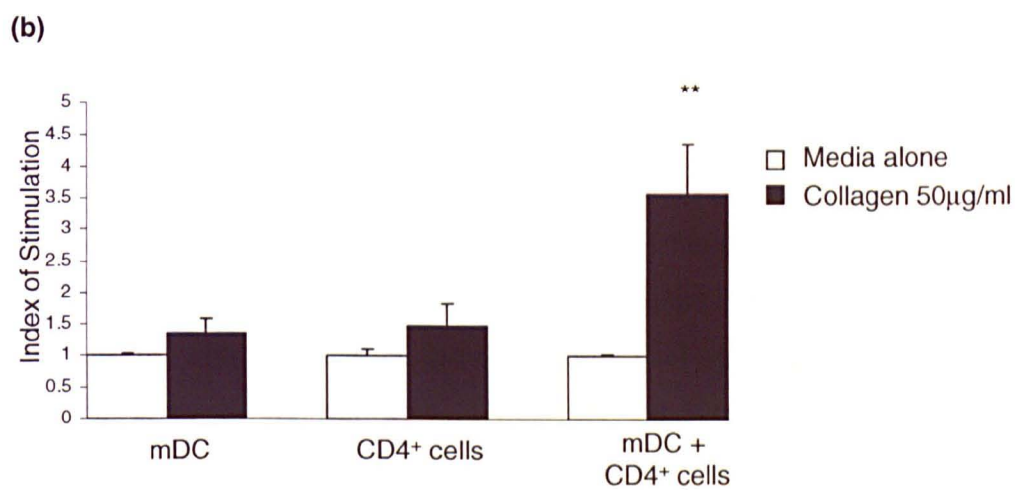
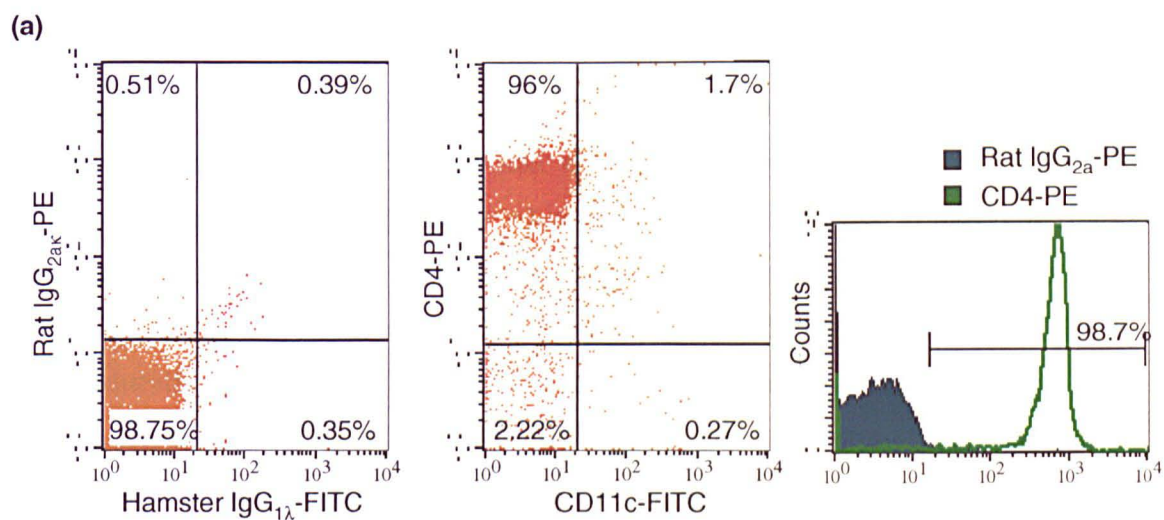




**Figure 6.5 mDC and pDC induce proliferation in collagen specific CD4<sup>+</sup> T cells.**

The phenotype of CD4<sup>+</sup> T cells purified from lymph nodes taken from DBA-1 mice undergoing collagen induced arthritis was confirmed by FACS (**a**, **b** and **c**) and revealed less than 2% contamination with CD11c<sup>+</sup> cells. The proliferation of the CD4<sup>+</sup> T cells following 72h incubation with either bone marrow derived mDC in media alone or containing 50µg/ml collagen (**d**) or bone marrow derived pDC in media alone or containing 50µg/ml collagen or pDC matured with 3.2µg/ml CpG ODN 1826 for 2h following incubation with collagen (**e**), was assessed by thymidine incorporation. Results are expressed as the stimulation index of an experiment carried out in triplicate. Results are representative of three replicable experiments.

\*<0.05 \*\*<0.01



(Fig. 6.5b). CII (50 $\mu$ g/ml) pulsed pDC also induced significant proliferation in CD4<sup>+</sup> T cells (Index of Stimulation, 12.96,  $p=0.001$ ) (Fig. 6.5c). Of interest, CpG ODN 1826 (3.2 $\mu$ g/ml) maturation of pDC prior to CD4<sup>+</sup> T cell co-culture, but after 6 hour pulse in CII (50 $\mu$ g/ml) did not further enhance CD4<sup>+</sup> T cell proliferation, but appeared instead to inhibit proliferation as compared to pDC that had not been matured. However, CD4<sup>+</sup> T cell proliferation was still significantly enhanced in these cultures compared to base-line (Index of Stimulation, 5.57,  $p=0.049$ ). This result was consistent with our foregoing data that indicated CpG ODN 1826 induced pDC maturation did not further enhance MHC/OVA-peptide induced fluorescence of hybridoma T cells (section 6.2.4). Since pDC macropinocytosis is poor, it is likely that additional maturation stimuli down-regulated pDC CII uptake as cells matured, thus actual antigen uptake may have been sub-optimal for subsequent immunogenic CD4<sup>+</sup> T cell expansion. The time of onset of CIA differs between individual DBA/1, therefore draining LN were harvested on different days placing restrictions on cell numbers. As a consequence of this technical restriction pDC and mDC were not assayed in parallel and therefore no direct comparison can be made between these two groups.

CIA in DBA/1 mice has been used extensively to investigate the relative contribution of different immune cell subsets to disease pathology and to evaluate the efficacy of different therapeutics therein. However, the contribution of the individual agents eliciting CIA (e.g. adjuvant components) are ill-defined, thus interpreting the mechanisms contributing directly to articular inflammation is difficult. To resolve this issue, this laboratory has developed new models of arthritis whereby the mechanisms inciting disease are more clearly defined, thus facilitating investigation of the immune cells contributing directly to pathology (697). One of these models established that activated mDC presenting CII peptide can induce arthritis upon transfer to naïve recipients (553), demonstrating their capacity to break self-tolerance. However, the ability of pDC to break self-tolerance, or not, was not addressed previously. The foregoing data indicated that both mDC and pDC could take-up, process and present CII. However, it also suggested the possibility that pDC would not be able to induce naïve CII specific CD4<sup>+</sup> T cell expansion alone. In addition, culture of bone marrow derived pDC proved difficult, with only relatively small numbers obtainable after microbead purification. We therefore decided that unlike mDC, bone marrow derived pDC pulsed *ex vivo* with CII and transferred to naïve DBA/1 mice

would be unlikely to induce arthritis. Furthermore, the technical difficulties associated with pDC culture precluded such an experiment since the number of mice and reagents required was prohibitive. pDC were, however, clearly efficient at expanding antigen experienced CD4<sup>+</sup> T cells, commensurate with an active role during the antigen-specific adaptive stage of arthritis. We therefore decided to analyse pDC contribution to a second murine model of arthritis developed by this laboratory using the pDC specific depleting antibody, 120G8.

### 6.3.6 T cell transfer model of arthritis

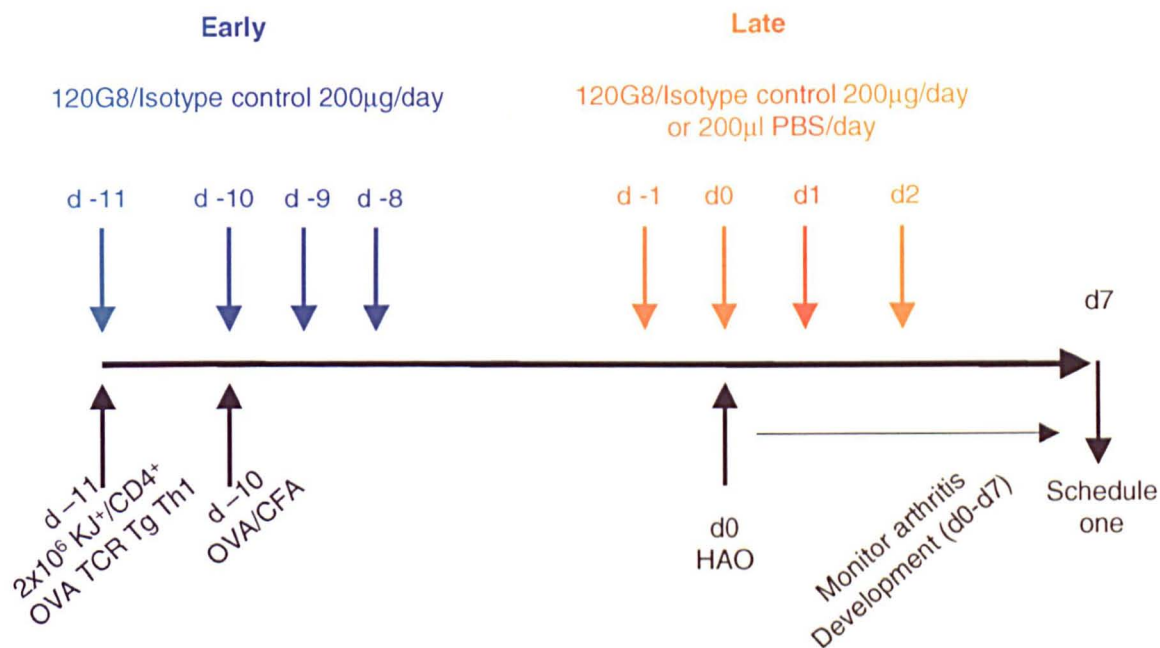
#### 6.3.6.1 Model

This laboratory has demonstrated that transfer of Th1, but not Th2 polarised, OVA-specific TcR Tg T cells to BALB/c recipient mice produces arthritis in joints proximal to the site of OVA inoculation (697). The model is outlined in figure 6.6. Briefly, at 11 days prior to challenge, 2x10<sup>6</sup> OVA TcR transgenic Th1 cells were transferred by i.v injection to all recipient female 6-8 week old BALB/c mice. One day following adoptive transfer, recipients received s.c injection of 100µg OVA in CFA. At day 0 all animals were injected peri-articular to the left ankle joint with 100µg of heat-aggregated OVA (HAO) in 50 µl of saline. The development of arthritis was monitored from day 0 to day 7 by measuring paw thickness with a dial caliper. At day 7 the *in vivo* experiment was concluded, mice sacrificed and hind limbs removed and fixed in 10% neutral-buffered formalin. Blood was collected for the analysis of serum antibody. The draining popliteal and inguinal lymph nodes were harvested for proliferation, cytokine and T cell analysis.

We decided to deplete pDC at either the priming stage or challenge stage of disease in order to estimate the kinetics of their relative contribution, if any, to pathology (Fig. 6.6). pDC were depleted by s.c. injection of 120G8 rat IgG<sub>1κ</sub> pDC depleting antibody and disease progression measured in comparison to animals given the irrelevant isotype control antibody YCATE.55.9.1 (Rat anti-Dog CD8 IgG<sub>1κ</sub>) or PBS. Using paw swelling as the primary outcome measure, we estimated that group sizes of 10 were required to have a 90% power of demonstrating significant increases at the 1% level in two sided Student's t test (mean paw diameter increases from 2.1 to 2.3 mm, with standard deviation of 0.11 mm). However, limits in the amount of 120G8 depleting antibody and isotype control

**Figure 6.6 Protocol for the depletion of pDC in the OVA TcR transgenic T cell transfer model of experimental arthritis.**

At 11 days (d-11) prior to challenge (d0)  $2 \times 10^6$  OVA TcR transgenic Th1 cells were transferred by i.v injection to all recipient female 6-8 week old BALB/c mice (n=42). One day following adoptive transfer (d-10) recipients received s.c injection of 100 $\mu$ g OVA in CFA. At 11 (d-11), 10 (d-10), 9 (d-9) and 8 (d-8) days prior to challenge some animals received 200 $\mu$ g of 120G8 rat IgG<sub>1k</sub> pDC depleting antibody (n=6) or 200 $\mu$ g of YCATE.55.9.1 rat anti-dog CD8 IgG<sub>1k</sub> irrelevant isotype control (n=6) i.p and are thus designated as the Early test groups. At day 0 all animals were injected s.c close to the left ankle joint with 100 $\mu$ g of heat-aggregated OVA (HAO) in 50 $\mu$ l of saline. At 1 day (d-1) prior to challenge, on the day of challenge (d0) and 1 (d1) and 2 (d2) days post-challenge animals that had not yet received pDC depleting antibody or isotype control were given 200 $\mu$ g of 120G8 rat IgG<sub>1k</sub> pDC depleting antibody (n=10) or 200 $\mu$ g of YCATE.55.9.1 rat anti-dog CD8 IgG<sub>1k</sub> irrelevant isotype control (n=10) i.p and are thus designated as the Late test groups. The remaining mice were given 200 $\mu$ l PBS i.p on d-1, d0, d1 and d2 (n=10) and are the control group. The development of arthritis was monitored from d0 to d7 by measuring paw thickness with a dial caliper. At d7 the *in vivo* experiment was concluded, mice sacrificed and hind limbs removed and fixed in 10% neutral-buffered formalin. Blood was collected for the analysis of serum antibody. The draining popliteal and inguinal lymph nodes were harvested for restimulation assays and T cell analysis.



120G8 Rat IgG<sub>1κ</sub> = pDC Depleting Antibody  
 Isotype control = YCATE.55.9.1 (Rat anti-Dog CD8 IgG<sub>1κ</sub>)

available placed restrictions on the number of animals we could examine. Since we were particularly interested in the disease outcome following pDC depletion at the challenge stage and we therefore decided to examine 10 mice per group at this stage, with smaller numbers examined at early depletion time points. A preliminary study of this model suggested that pDC were more likely to participate in disease pathology at the challenge stage. Furthermore, the challenge stage was considered more relevant to human disease since patients presenting in the clinic already have established arthritis.

There were therefore 5 groups of animals: PBS injection (n=10) (1, positive control); 120G8 pDC depletion at the time of OVA Tg T cell transfer (n=6) (2, early pDC depletion); YCATE.55.9.1 isotype control at the time of OVA Tg T cell transfer (n=6) (3, early isotype control); 120G8 pDC depletion at the time of OVA challenge (n=10) (4, late pDC depletion); and YCATE.55.9.1 isotype control at the time of OVA challenge (n=10) (5, late isotype control).

#### **6.3.6.2 120G8 depletion of pDC**

To confirm pDC depletion after 4 s.c injections of 120G8 (200 $\mu$ g) 1 animal from each group (120G8, YCATE.55.1 irrelevant isotype control antibody or PBS) was sacrificed and LN and spleen harvested. pDC in single cell suspensions were detected by CD11c<sup>lo</sup>/B220<sup>+</sup> and mPDCA-1<sup>+</sup> staining as analyzed by FACS. pDC were depleted from BALB/c by 120G8 (Fig. 6.7b and e), but not from PBS (Fig. 6.7a and d) or isotype control group (Fig. 6.7c and f). CD11c<sup>lo</sup>/B220<sup>+</sup> pDC were not depleted in the PBS group (0.8% of cells, Fig. 6.7a) or isotype control group (0.65% of cells, Fig. 6.7c), but were depleted by ~70% in the 120G8 group (0.36% of cells, Fig. 6.7b). Of interest, pDC as analyzed by mPDCA-1<sup>+</sup> staining were, again, not depleted in PBS (0.46% of cells, Fig. 6.7d) or isotype control groups (0.4%, Fig. 6.7f), but were almost completely depleted from the 120G8 group (0.06%, Fig. 6.7e). The latter is a more specific measurement of pDC, since mPDCA-1 is expressed by pDC only, but B cells are B220<sup>+</sup>/CD11c<sup>-</sup> and mDC are B220<sup>-</sup>/CD11c<sup>+</sup>.

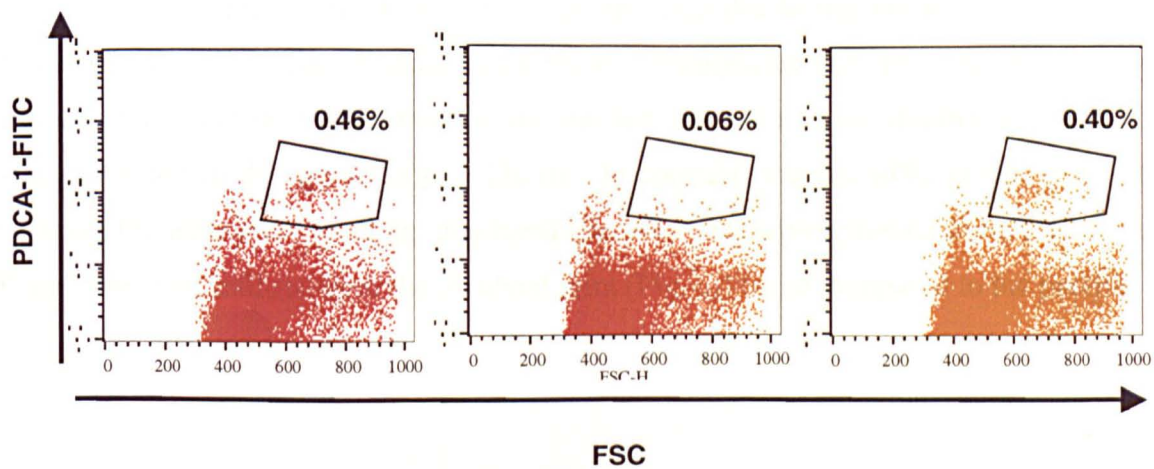
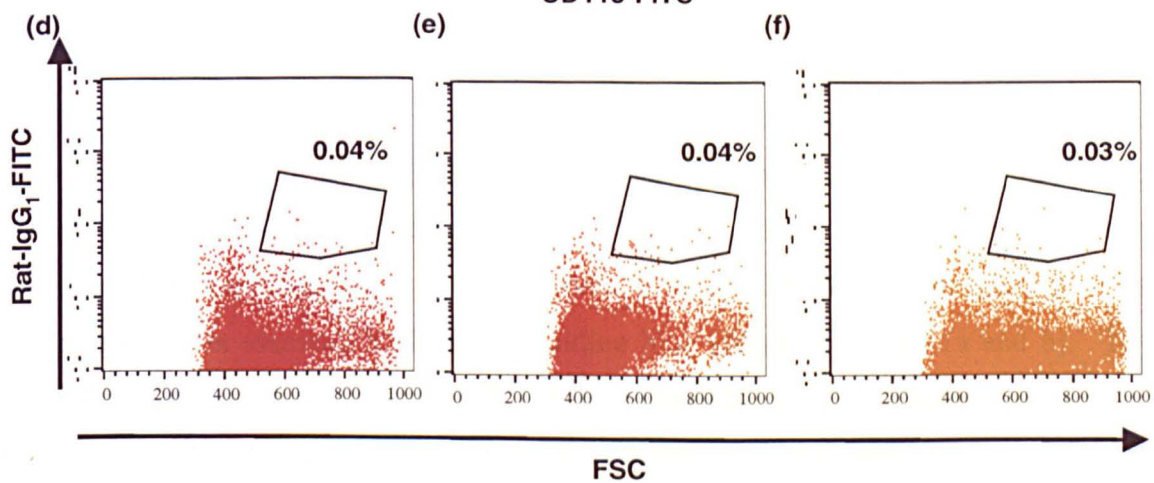
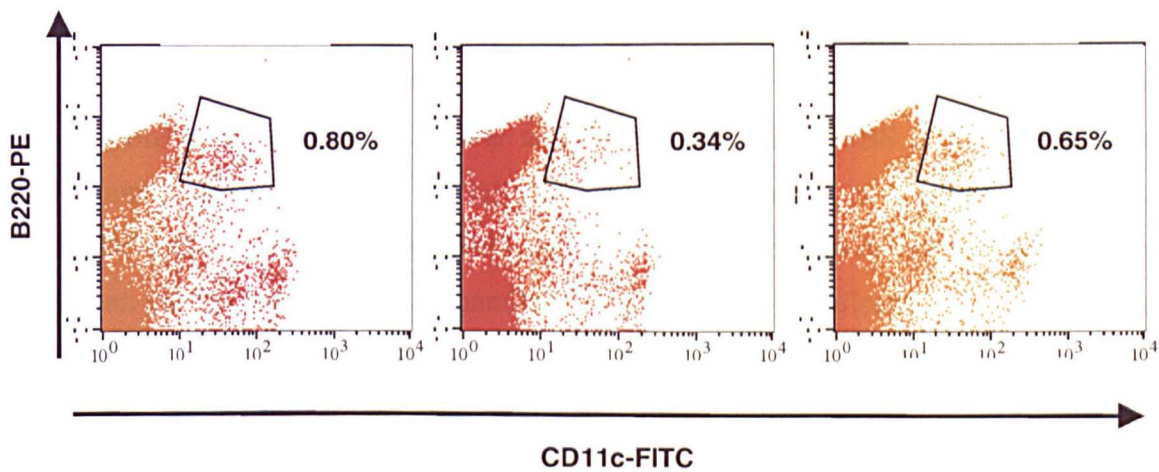
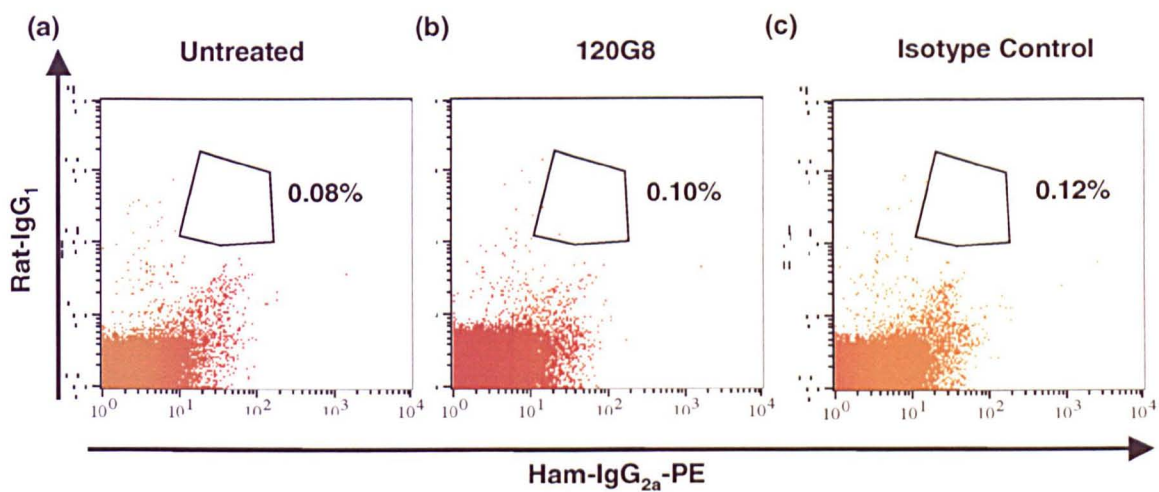
#### **6.3.6.3 Phenotype of OVA TCR Tg T cells**

We purified naïve CD4<sup>+</sup> T cells from pooled LN cells harvested from DO11.10 mice by negative selection. T cell differentiation was induced by co-culture with mitomycin C treated APC and 0.3 $\mu$ M OVA<sub>323-339</sub>. Th1 phenotype was induced by supplementing



### **Figure 6.7 120G8 depletion of pDC.**

The pDC and mDC in spleen and lymph nodes harvested on day 5 from Balb-c mice given 200 $\mu$ l PBS (**a** and **d**), 200 $\mu$ g 120G8 pDC depleting antibody (**b** and **e**) or 200 $\mu$ g Rat anti-Dog CD8 IgG<sub>2a</sub> irrelevant isotype control (**c** and **f**) i.p per day for 4 days, was determined by double staining with B220 and CD11c or the relevant isotype controls and confirmed with the pDC specific PDCA-1 or relevant isotype controls. pDC are B220<sup>+</sup>/CD11c<sup>lo</sup>/PDCA-1<sup>+</sup>, mDC are B220<sup>-</sup>/CD11c<sup>high</sup>/PDCA-1<sup>-</sup> and B cells are B220<sup>+</sup>/CD11c<sup>-</sup>/PDCA-1<sup>-</sup>. FACS analysis of spleen samples is shown.



cultures with IL-12 (5ng/ml) and anti-IL-4 mAb (10µg/ml). After 3 days of culture, cells were harvested for transfer to recipient BALB/c. To ensure transfer of  $2 \times 10^6$  OVA TcR transgenic cells to recipients, their percentage in culture was detected by CD4<sup>+</sup>/KJ1.26<sup>+</sup> staining and FACS analysis and was routinely >53% (Fig. 6.8a). Intracellular staining and FACS analysis demonstrated that >66% of Tg T cells expressed IFN-γ, confirming the Th1 phenotype (Fig. 6.8b).

#### **6.3.6.4 Clinical effects of cellular depletion**

The swelling of the left hind paw (site of HAO injection) was measured using dial calipers. Transfer of Th1 cells into recipient mice induced swelling in all groups, first evident at day 1, and peaking at day 2, after challenge with HAO. The early pDC depletion group displayed more swelling that was slower to resolve, than did the PBS or parallel isotype control groups, significant at day 1 (PBS vs Early pDC depletion,  $p = 0.008$ ) (Fig. 6.9a). The late pDC depletion group developed lower levels of inflammation that were quicker to resolve, than did the PBS control or parallel isotype control, significant at day 2 (PBS vs Late pDC depletion,  $p = 0.042$ ) (Fig. 6.9b). There was no appreciable swelling in the right hind paws in any of the groups (Fig. 6.10a and b) indicating no contra-lateral disease transfer.

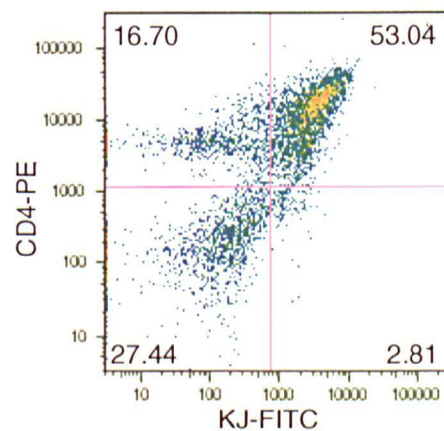
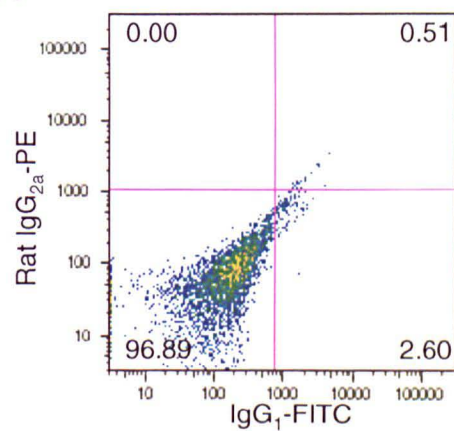
#### **6.3.6.5 Histological analysis**

We performed sequential histological analyses on left hind paws removed at sacrifice and fixed in 10% neutral-buffered formalin. The PBS control group exhibited a modest infiltration of inflammatory cells to the involved joint and a partial to moderate level of cartilage erosion as assessed by loss of toluidine blue staining (Fig. 6.11a and b). The early and late isotype controls displayed similar inflammatory cell infiltration and loss of cartilage integrity, comparable to the PBS group (6.11e-f and i-j respectively). Unexpectedly, the early pDC depletion group, although displaying the most swelling, as determined using dial calipers, exhibited a lower inflammatory cell infiltrate than did the parallel isotype control or PBS control groups, and there was only minimal evidence of cartilage erosion in this group (Fig. 6.11c-d). In contrast, the late pDC group, although displaying the least joint swelling, displayed the most extensive synovial hyperplasia and inflammatory cell infiltration to the involved joint (Fig. 6.11g) as compared to the parallel

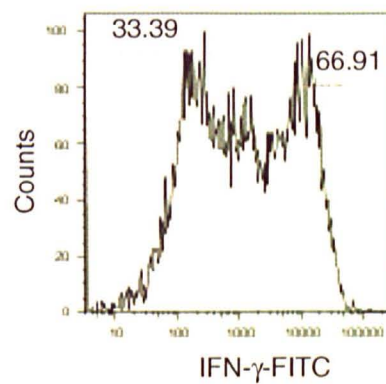
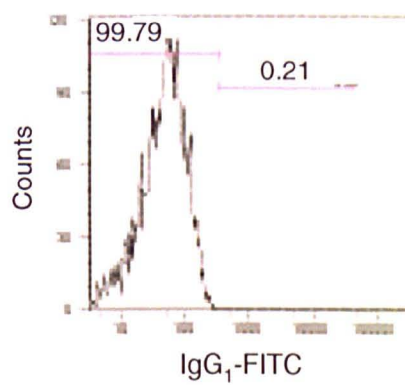
**Figure 6.8 Th1 phenotype of KJ1.26<sup>+</sup>/CD4<sup>+</sup> OVA specific transgenic T cells.**

CD4<sup>+</sup> T cells from pooled LN cells DO11.10 mice were purified by negative selection and cultured with mitomycin-C treated APC in the presence of 0.3  $\mu$ m OVA + 5ng/ml IL-12 + 10 $\mu$ g/ml anti-IL-4 mAb for 3 days in order to drive a Th1 skewed expansion of TcR OVA specific transgenic T cells. Prior to transfer to recipient mice, the percentage of TcR OVA specific transgenic T cells in culture was determined by double staining with KJ1.26 and CD4 and analysing by FACS (a) and their Th1 phenotype confirmed by intracellular IFN- $\gamma$  staining of KJ1.26<sup>+</sup>/CD4<sup>+</sup> cells (b). A total of 2x10<sup>6</sup> TcR OVA specific transgenic T cells were injected i.v into BALB/c recipients.

(a)



(b)

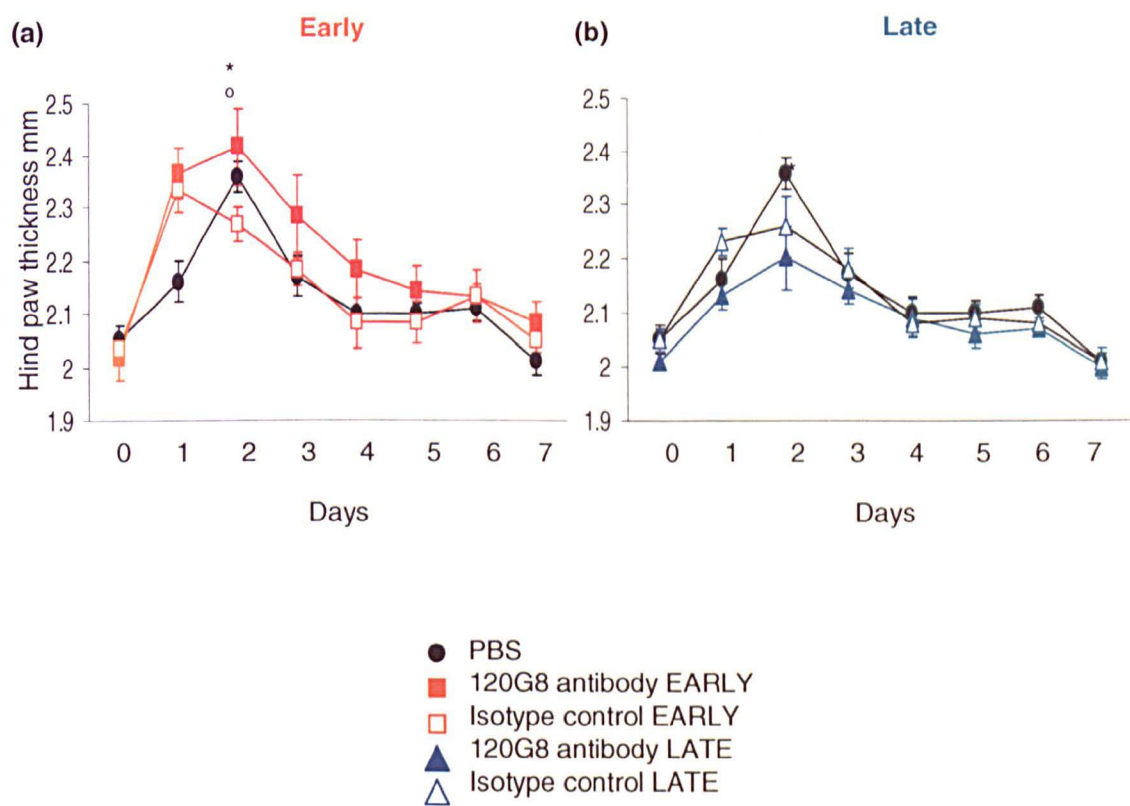


**Figure 6.9 Left hind paw swelling.**

The left hind paw thickness in mm of animals was measured using dial calipers just before challenge with HAO and every day thereafter for 7 days (d0-d7). Graphs show the swelling in animals given early injections of 120G8 pDC depleting antibody or isotype control as compared to control mice (a) and the swelling in animals given late injections of 120G8 pDC depleting antibody or isotype control as compared to control mice (b).

(a) day 1, \*\* PBS vs pDC deplete = 0.008, ° PBS vs isotype control = 0.011

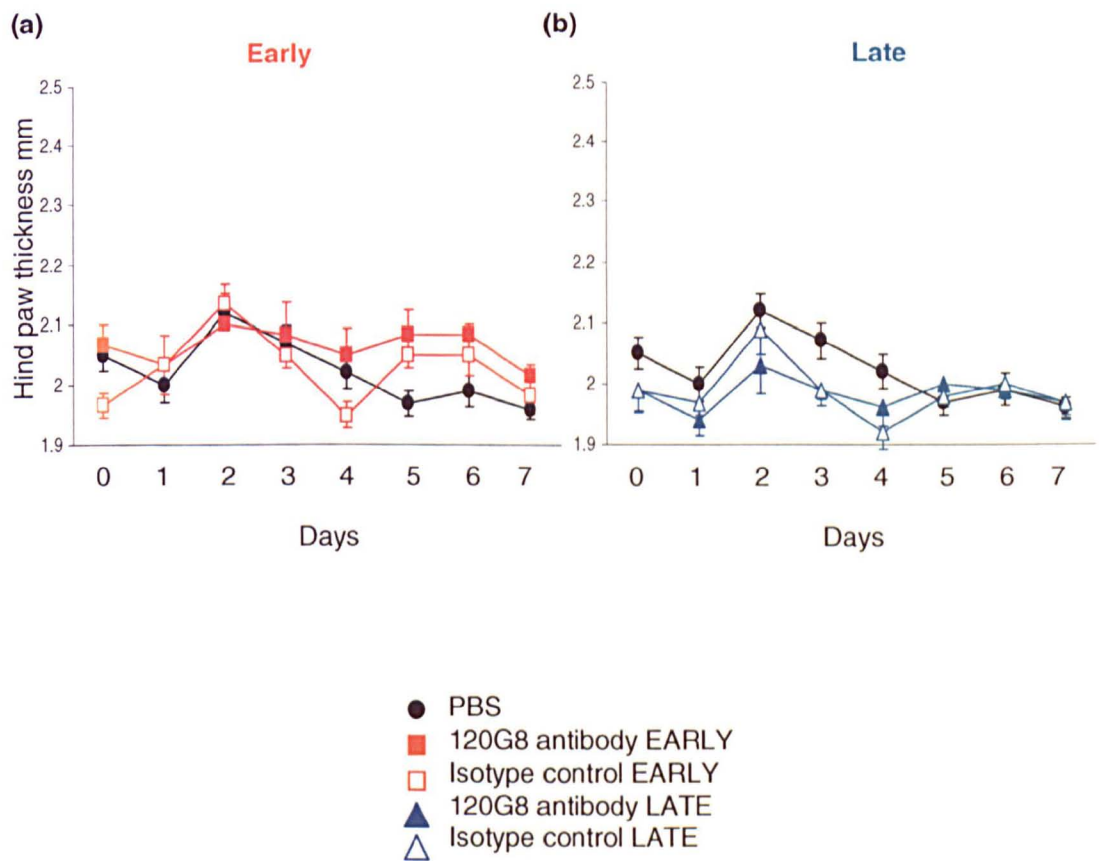
(b) day 2, \* PBS vs pDC deplete = 0.042



### **6.10 Right hind paw swelling.**

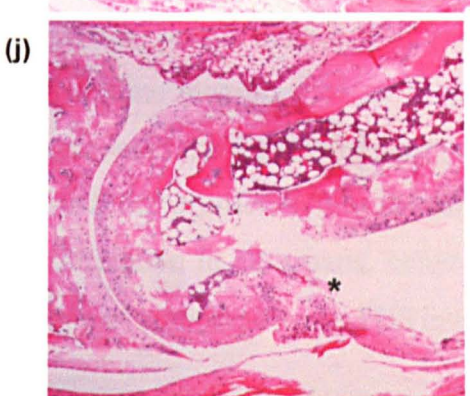
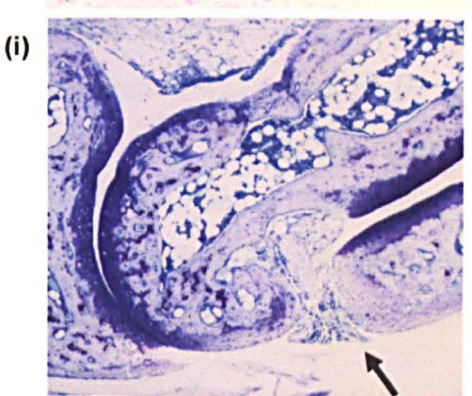
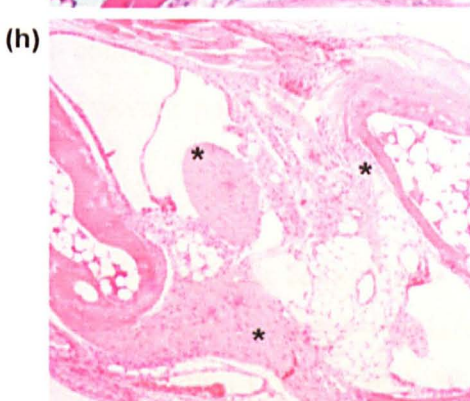
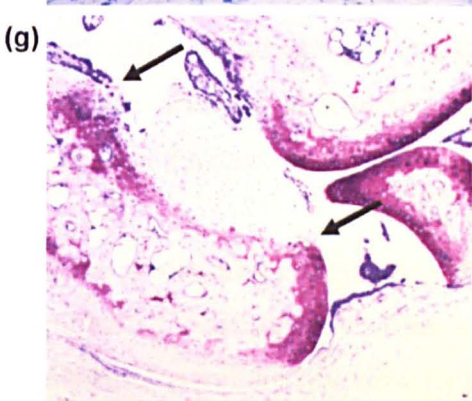
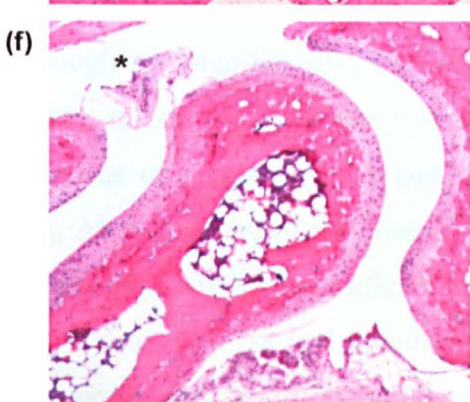
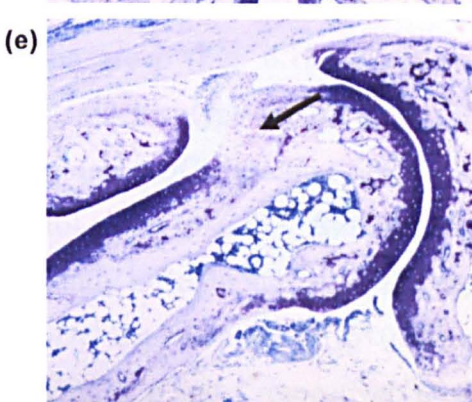
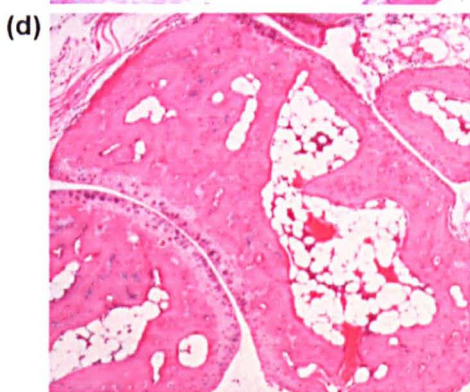
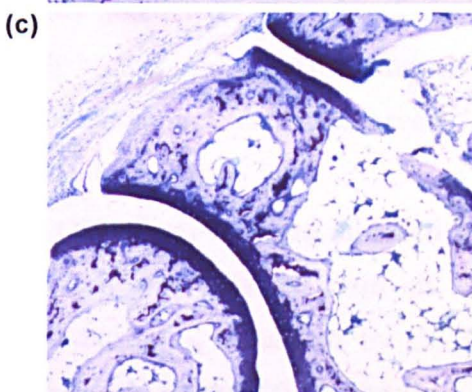
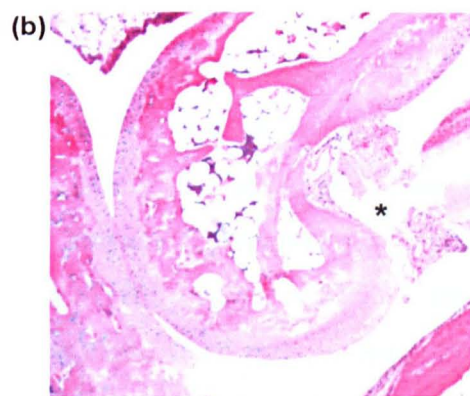
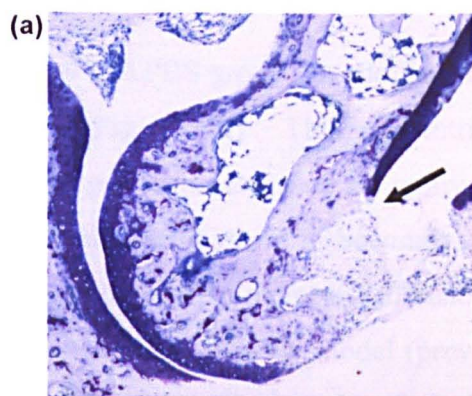
The thickness of the right hind paw in mm of animals was measured using dial calipers just before challenge with HAO and every day thereafter for 7 days (d0-d7). Graphs show the swelling in animals given early injections of 120G8 pDC depleting antibody or isotype control as compared to control mice (**a**) and the swelling in animals given late injections of 120G8 pDC depleting antibody or isotype control as compared to control mice (**b**).





### **Figure 6.11 Histological analysis of arthritis in recipient mice.**

Sections of the left hind limbs were taken and stained with toluidine blue (**a, c, e, g, i**) or haematoxylin and eosin (**b, d, f, h, j**). The ankle joints of PBS recipient mice demonstrated a lightened toluidine blue staining (**a**) indicative of cartilage erosion (↑) and some synovial hyperplasia and inflammatory cell infiltration (\*) (**b**). Recipients of early pDC depleting antibody demonstrated strong toluidine staining (**c**) indicative of a normal cartilage integrity and little inflammatory cell infiltration (**d**) while the early isotype control resembled the PBS recipient group (**e** and **f**). The ankle joints of late pDC depleting antibody recipient mice demonstrated reduced toluidine blue staining indicative of extensive cartilage and bone erosion (**g**), increased synovial hyperplasia and inflammatory cell infiltration (**h**), while the late isotype control group demonstrated comparatively less severe inflammation, resembling the PBS recipient group (**i** and **j**). One typical example from each group is shown.



isotype control and PBS group. In addition, there were moderate levels of cartilage erosion in this group (Fig. 6.11h). This was intriguing for a number of reasons. Firstly, it suggested that paw swelling used by many as an indicator of disease, is not necessarily appropriate in all murine models. Secondly, the data indicated that depletion of pDC at the challenge stage of disease increased disease severity. Thirdly however because histology was being assessed early in the model (previously assessed at 14 days) it is possible that the differences noted were not of real significance (noting that hind limb swelling had settled largely by this time point). Further studies are required to characterize the effects on the kinetics of histologic changes observed.

#### **6.3.6.6 Anti-OVA, anti-collagen and anti-IgG<sub>2a</sub> antibody production *in vivo***

At day 7, serum was obtained from all animals and serum levels of anti-OVA, anti-collagen and anti-IgG<sub>2a</sub> measured by ELISA. As expected, all animals were able to produce high levels of anti-OVA Abs (Fig. 6.12a and b). Anti-collagen Ab in the early pDC depletion group (Fig. 6.12c) was lower, although not significantly, than the PBS control and parallel isotype control group. In contrast, anti-collagen Ab was significantly higher in the late pDC depletion group (Fig. 6.12d) as compared to PBS and parallel isotype control group ( $p < 0.01$ ). Similarly, anti-IgG<sub>2a</sub> Ab (a measure of rheumatoid factor) were significantly lower in the early pDC depletion group ( $p < 0.05$ ) but significantly higher in the late pDC depletion group ( $p < 0.05$ ) as compared to the PBS and parallel isotype controls (Fig. 6.12 e and f respectively). Antibody production from the isotype control groups did not differ significantly from the PBS control. Together these data are consistent with the histological data indicating more severe pathology in the late pDC depletion group than the early pDC depletion group.

#### **6.3.6.7 Cytokine profile and cellular proliferation of OVA-specific cells *ex vivo***

Draining popliteal LN were removed from all mice at 7 day post challenge with HAO and single-cell suspensions cultured *in vitro* with or without 1 mg/ml OVA or with 50 µg/ml CII. Supernatants were harvested at 72 hours and cytokine profile measured by multiplex analysis. Proliferation was measured by tritiated thymidine incorporation at 96 hours of culture (Fig. 6.13a and b). Little proliferation was apparent in the absence of stimulation in all groups. *In vitro* culture in the presence of OVA induced proliferation to similar levels in all groups. Of interest, collagen also induced proliferation in all groups, however this was significantly higher in the late pDC depletion group as compared to the PBS control

**Figure 6.12 Serum anti-OVA Ab, anti-collagen Ab and anti-IgG<sub>2a</sub> Ab titration.**

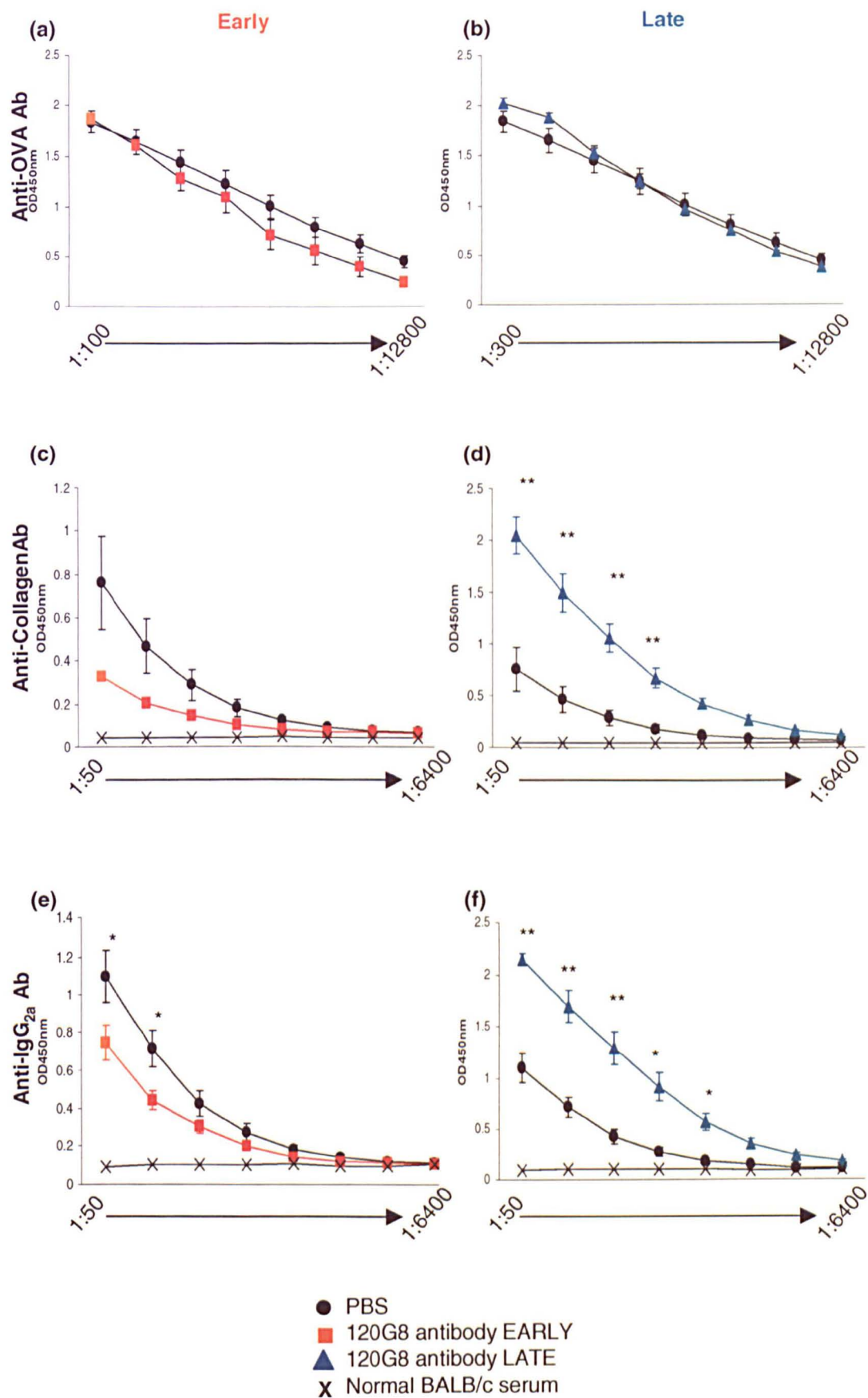
Serum anti-OVA IgG<sub>2a</sub> (a and b), anti-collagen IgG<sub>2a</sub> (c and d) and anti-IgG<sub>2a</sub> (e and f) Ab levels were measured on day 7 by ELISA by doubling serum titration. Serum levels were measured for each individual animal per group (PBS control n=10, early pDC depletion n=6, early isotype control n=6, late pDC depleting antibody n=10 and late isotype control n=10) with results presented as mean +/- SEM. The serum antibody titres of isotype controls did not differ significantly from controls and are not shown.

(d) 1:50, \*\* PBS vs pDC deplete = 0.003, 1:100, \*\* PBS vs pDC deplete = 0.003, 1:200, \*\* PBS vs pDC deplete = 0.002, 1:400, \*\* PBS vs pDC deplete = 0.005.

(e) 1:50, \* PBS vs pDC deplete = 0.043, 1:100, \* PBS vs pDC deplete = 0.044.

(f) 1:50, \*\* PBS vs pDC deplete = 0.001, 1:100, \*\* PBS vs pDC deplete = 0.002, 1:200, \*\* PBS vs pDC deplete = 0.004, 1:400, \* PBS vs pDC deplete = 0.011, 1:800, \* PBS vs pDC deplete = 0.045.





**Figure 6.13 *Ex vivo* cellular proliferation.**

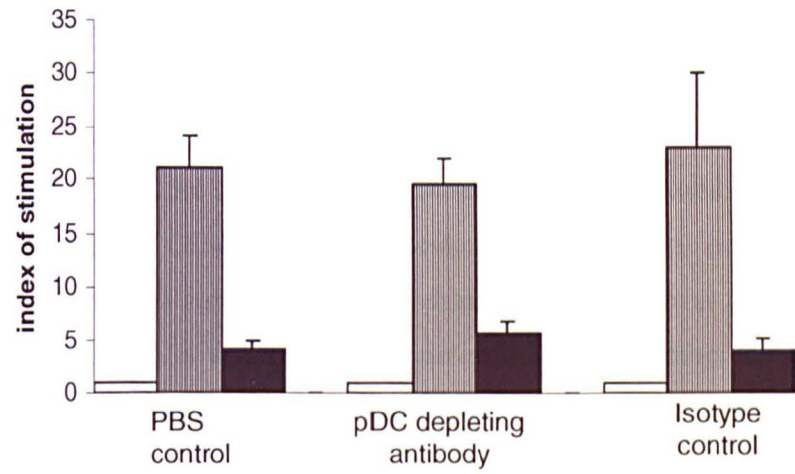
Draining lymph nodes were collected at day7 and cultured separately for each individual animal with medium alone, 1mg/ml OVA or 50µg/ml collagen II. [<sup>3</sup>H] Thymidine incorporation was measured at 96h. Results are presented as the mean stimulation index +/- SEM for each group (PBS control n=10, early pDC depletion n=6, early isotype control n=6, late pDC depleting antibody n=10 and late isotype control n=10).

\* control vs late pDC depleting antibody = 0.02831

° late pDC depleting antibody vs late isotype control = 0.01185

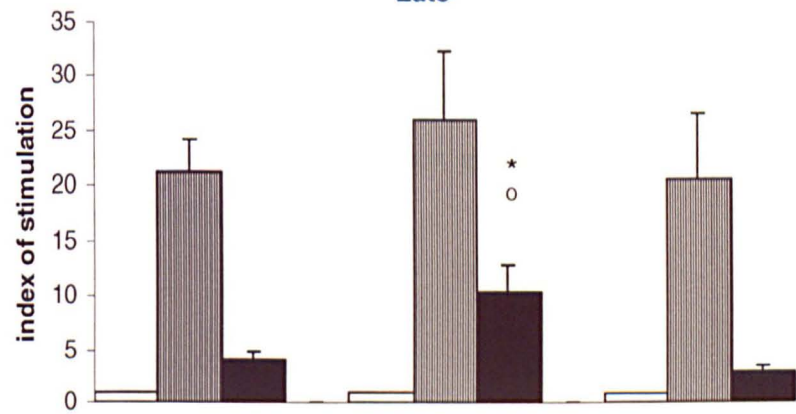
(a)

Early



(b)

Late



Media  
OVA 1mg/ml  
Collagen 50µg/ml



and parallel isotype control (PBS vs Late pDC depletion,  $p = 0.02831$ , Isotype control vs Late pDC depletion,  $p = 0.01185$ ) (Fig. 6.13b). Draining LN cultures taken from all groups continued to produce IFN- $\gamma$  after stimulation with OVA (Fig. 6.14a and b). OVA stimulation also induced moderate levels of IL-5 in all cultures (Fig. 14c and d), but relatively low levels of IL-4, IL-10 and GM-CSF (Fig. 14e-j). Cytokine secretion from cells taken from the late pDC depletion group tended to be greater than PBS control or parallel isotype control, but this was significant for IL-5 only ( $p = 0.049$ ).

#### **6.3.6.8 Profile of CD4<sup>+</sup> T cells in draining LN**

In order to determine if any of the differences we observed thus far could be attributed to differences in the numbers of CD4<sup>+</sup> T cells expanded in each group, or the phenotype thereof, we analysed single-cell suspensions from draining LNs taken at day 7 for CD4<sup>+</sup> and KJ.1<sup>+</sup> expression (Fig. 6.15a and b) and we determined the number of Tregulatory (Treg) cells therein by co-staining of CD4 with intracellular Forkhead-box (Fox) p3, a Treg specific marker.

By day 7, there were minimal KJ1.26<sup>+</sup> OVA<sub>323-339</sub> peptide-specific TCR Tg T cells as assessed by CD4<sup>+</sup> /KJ1.26<sup>+</sup> staining and FACS analysis (Fig. 6.15a). The overall expansion of CD4<sup>+</sup> cells was comparable in all groups at ~50% of all cells in the draining LNs. Similarly, Tregs comprised 7.4-8.4% of all CD4<sup>+</sup> cells and there were no differences between groups (Fig. 6.16). Together these results indicate that the differences in disease severity observed in the early and late pDC depletion groups could not be explained by differing levels of CD4<sup>+</sup> T cells, or Treg cells.

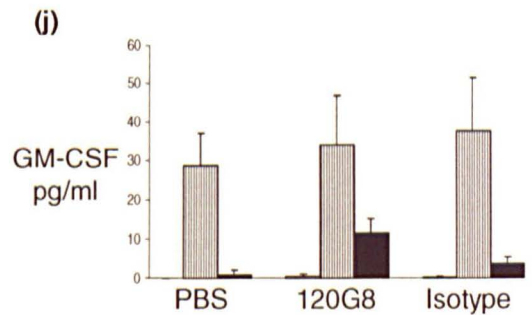
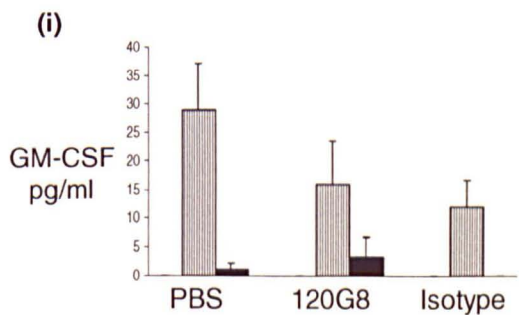
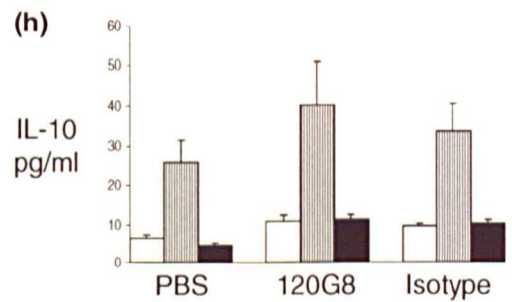
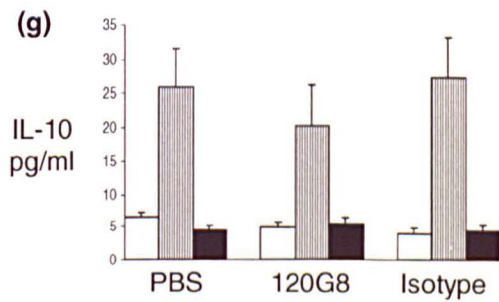
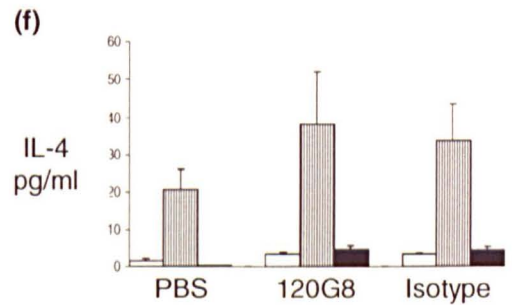
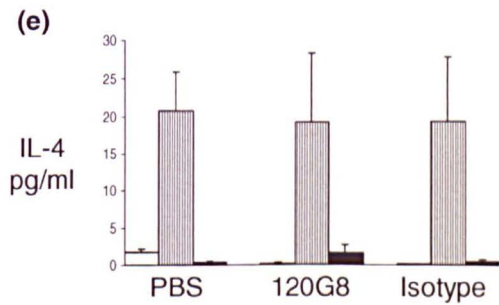
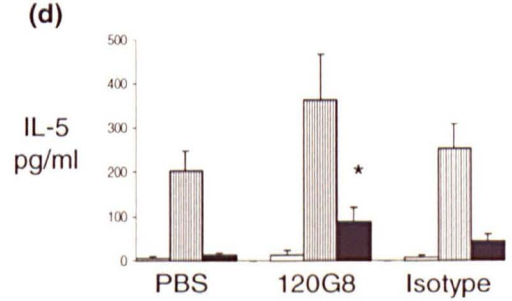
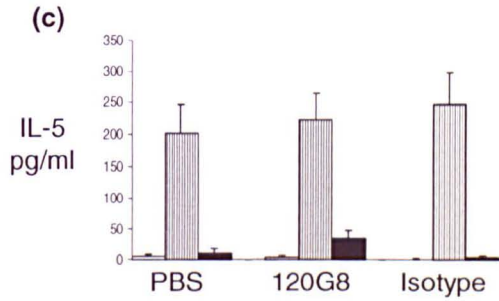
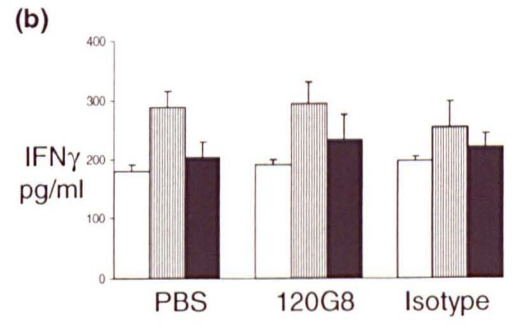
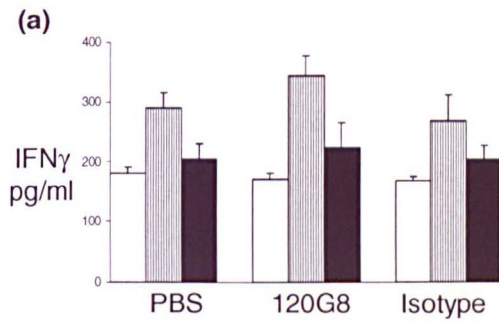
**Figure 6.14 *Ex vivo* cytokine profile of lymph nodes.**

Draining lymph nodes were collected at day7 and cultured separately for each individual animal (PBS control n=10, early pDC depletion n=6, early isotype control n=6, late pDC depleting antibody n=10 and late isotype control n=10) in triplicate, with medium alone, 1mg/ml OVA or 50µg/ml collagen II. The levels of the cytokines IFN-γ (a and b), IL-5 (c and d), IL-4 (e and f), IL-10 (g and h) and GM-CSF (i and j) in the culture supernatant at 72h were measured by Luminex analysis. The results represent the mean +/- SEM.

(b) \* control vs late pDC depleting antibody = 0.049

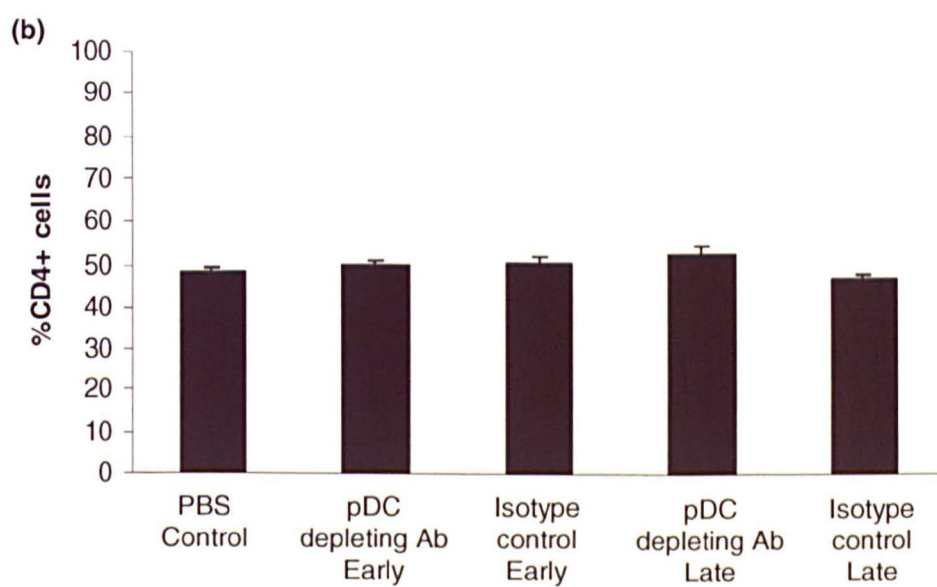
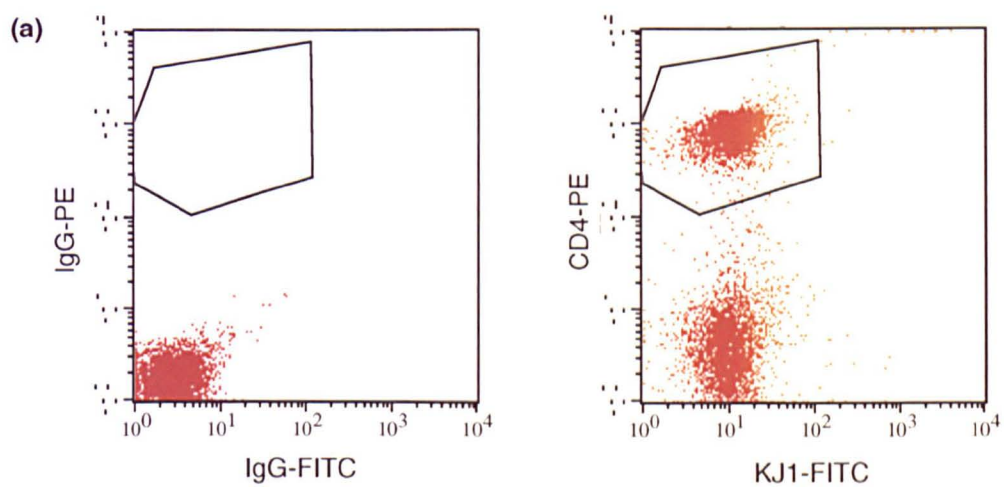
Early

Late



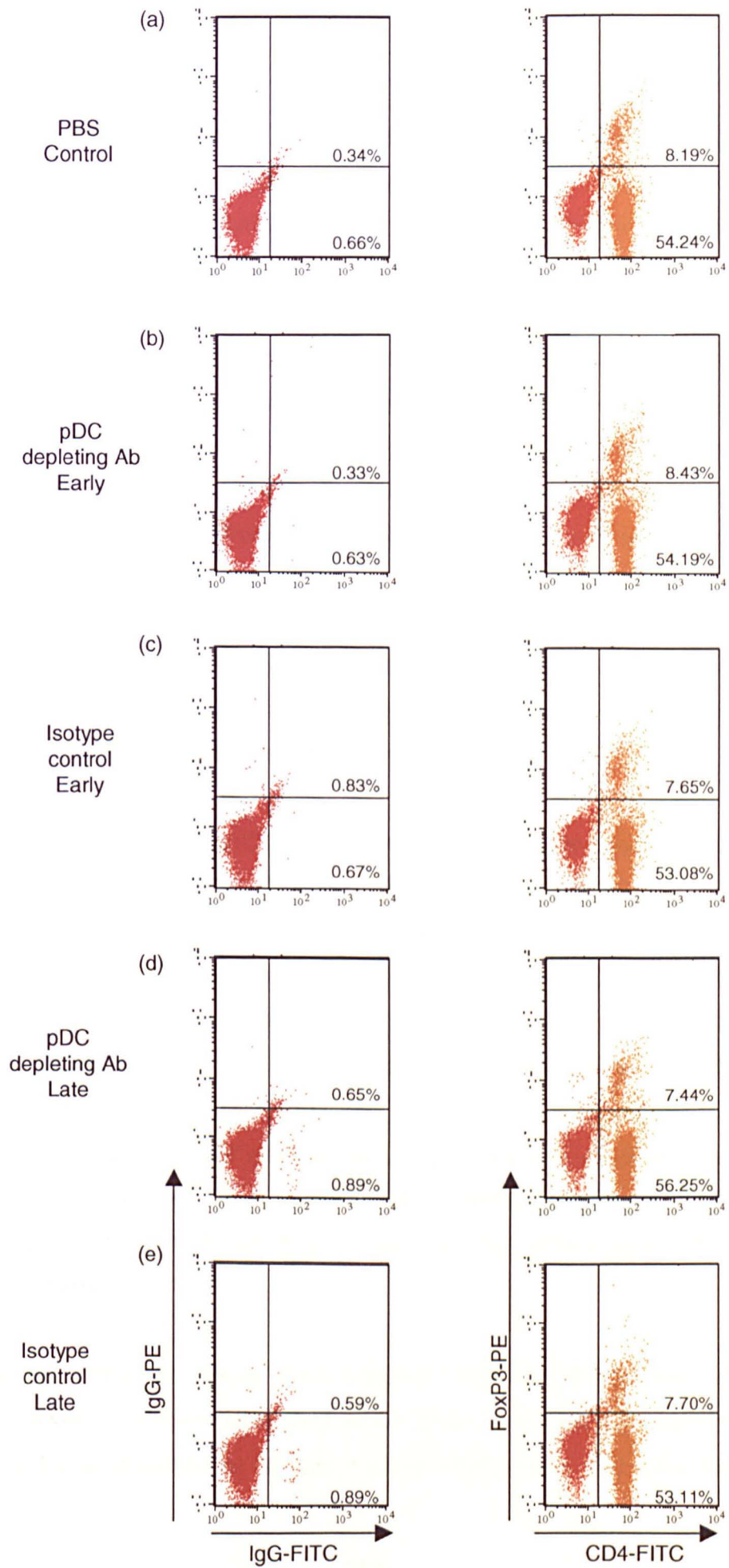
**Figure 6.15 Percentage of CD4<sup>+</sup> cells in draining lymph nodes.**

The percentage of CD4<sup>+</sup>/KJ1.26<sup>+</sup> (TcR OVA specific transgenic T cells) in the draining lymph nodes of mice receiving PBS, early pDC depleting antibody, early isotype control, late pDC depleting antibody and late isotype control was measured at day 7 by FACS analysis. The percentage of CD4<sup>+</sup>/KJ1.26<sup>+</sup> in all groups was minimal (<0.2%) (a). One representative FACS plot is shown. CD4<sup>+</sup>/KJ1.26<sup>-</sup> cells were expanded in the draining lymph nodes from all groups (b). Results represent the mean +/- SEM for three animals per group.



**Figure 6.16 Foxp3<sup>+</sup> Treg analysis of draining lymph nodes.**

The percentage of CD4<sup>+</sup>/Foxp3<sup>+</sup> (Tregs) in the draining lymph nodes of mice receiving PBS (a), early pDC depleting antibody (b), early isotype control (c), late pDC depleting antibody (d) and late isotype control (e) was measured at day 7 by FACS analysis. The percentage of CD4<sup>+</sup>/Foxp3<sup>+</sup> cells in all groups was comparable. One representative FACS plot from each group is shown.



## 6.4 Discussion

Dendritic cells (DC) are critical to the generation and maintenance of central and peripheral tolerance, as well as the priming of innate and adaptive immunity. As such, DC play an essential role in the initiation and perpetuation of inflammatory arthritis as loss of self-tolerance is a critical component of autoimmune disease. Maffia et al. recently reported that adoptive transfer of Th1 differentiated OVA TCR Tg T cells induced arthritis in recipient mice following OVA immunization (697). It was subsequently postulated that local regulatory mechanisms that maintain self-tolerance, particularly those sustained by DC, could be overwhelmed by a strong Th1 response to irrelevant antigen, enabling the production of autoreactive T cells. Similar murine models have been described that have demonstrated that respiratory viruses can break inhalation tolerance to harmless antigens (698, 699). Moreover, numerous infectious agents including bacteria, mycobacteria, viruses and parasites have been implicated in triggering and exacerbating RA and PsA (211-214, 221). We have now analyzed the contribution of pDC to the aetiology of this model and provide evidence indicating that pDC may be important in supporting the suppression of autoimmune inflammatory arthritis.

We first established that both pDC and mDC are expanded in the draining lymph node of mice undergoing collagen-induced arthritis (CIA). That pDC exceeded mDC was expected as pDC comprise the most abundant DC population in the lymph node owing to their high level expression of lymph node homing chemokine receptors including CD62L, CXCR3 and CCR7 (442). This was commensurate with our previous hypothesis that reduced pDC and mDC numbers in the peripheral circulation of RA and PsA patients could, at least in part, be explained by lymph node directed migration, in addition to the observed infiltration to the target inflamed synovial tissue and psoriatic skin lesions (Chapter 3 and 4), particularly since reduced PB DC subsets could be correlated with active inflammation. However, this did not provide information regarding the immunogenic potential of the migrating DC subsets.

The competence of pDC to take up whole antigens, other than viruses, and elicit antigen-specific responses is controversial as they do not phagocytose, process or load antigens to MHC molecules as efficiently as do their myeloid counterparts. We have demonstrated



here that pDC are capable of processing and presenting OVA peptide to OVA TCR Tg T cells. Similar observations have been made by de Heer et al, who found however that unlike mDC, OVA-loaded pDC could not induce proliferation of naïve TCR-transgenic OVA specific T cells *ex vivo* (700). While it is generally accepted that pDC can expand antigen-experienced T cells (453, 690), their ability to expand naïve T cells is a more contentious issue, particularly as pDC have only low MHC II and co-stimulatory receptor expression compared to mDC, even when terminally matured. We observed that although bone marrow derived mDC and pDC could both elicit collagen-specific expansion of CD4<sup>+</sup> T cells isolated from the draining lymph nodes of DBA/1 mice undergoing CIA, only mDC elicited low level proliferation to collagen in lymph node derived CD4<sup>+</sup> T cells isolated from naïve DBA/1 mice. It is possible that the proliferation induced in mDC/T cell cultures was indicative of a small number of self-reactive T cells in culture. I-A<sup>q</sup> haplotype DBA/1 mice are highly susceptible to CIA and it has been shown that expansion of antigen-specific CD4<sup>+</sup> T cells after intradermal injection of CII emulsified in complete Freund's adjuvant (CFA) occurs early *in vivo*, before the onset of arthritis (696). We have also observed that during CIA, up to 10% of animals develop arthritis (as assessed by paw swelling) prior to intraperitoneal CII challenge (unpublished communication from Dr J.A. Gracie). In addition to this, it has been suggested that collagen may deliver a direct maturation stimulus to mDC possibly through TLR4 signaling (694, 695), thus enhancing their immunogenic potential. While murine mDC mature rapidly to TLR4 stimulation, murine pDC are only weakly responsive (701) and their human pDC counterparts do not respond to TLR4 agonists at all (490, 499). It is therefore unlikely that collagen-loaded pDC would be immunogenic in the absence of external inflammatory stimuli. However, we observed that TLR 9 stimulation with CpG ODN did not enhance pDC antigen-specific stimulation of T cells. Together the data supports a central role for mDC, but not necessarily pDC, in the initial naïve CD4<sup>+</sup> T cell expansion phase of CIA. It has been postulated that pDC act complementary to mDC, directing the differentiation of unpolarized T cells previously expanded by mDC (453) - this possibility requires further investigation. Of particular interest, it has been indicated in several murine models that pDC are important to the maintenance of tolerance (700, 702).

The direct contribution of mDC to the pathogenesis of various murine models of arthritis has been reported earlier by us (553) and others (550, 551), however there has as yet been no attempt to analyze the relative contribution of pDC to arthritic pathology in a murine

model. We next performed experiments aimed at elucidating the potential pathogenic role of pDC in a murine model of arthritis. Technical restrictions precluded the expansion of sufficient numbers of bone marrow derived pDC to enable *in vivo* transfer experiments. We also chose not to utilize the collagen-induced arthritis in DBA/1 model as, although it was informative in our *in vitro* analyses, significant problems exist in interpreting *in vivo* disease mechanisms attributable to the multifactorial contribution of eliciting agents. In addition, we were specifically interested in analyzing DC subsets, and thus concluded that this would be better achieved using the Th1 OVA TcR Tg adoptive transfer model of arthritis, particularly as autoimmunity is postulated to arise from a DC specific breach of self-tolerance (697). We therefore chose to use the pDC specific antibody 120G8 to deplete pDC at the time of adoptive T cell transfer in comparison to pDC depletion at the time of OVA challenge in the OVA TCR Tg adoptive transfer model of arthritis.

We first observed that all animals, whether depleted of pDC or not, developed transient inflammation at the site of OVA immunization. However, the magnitude of this inflammation and the kinetics of its resolution were significantly altered between groups. Mice from which pDC had been depleted at the time of adoptive T cell transfer had significantly more footpad swelling that was slower to resolve than did control animals or mice from which pDC had been depleted at the later time of OVA challenge. Indeed, the later pDC depleted group had significantly less footpad swelling at early time points than did control animals. However, footpad swelling was not indicative of destructive joint pathology. Histological examination revealed that late pDC depleted animals exhibited more extensive synovial hyperplasia, inflammatory cell infiltration and cartilage erosion in the involved joint than did mice from the early pDC depletion or parallel control groups. Commensurate with this, animals within the later pDC depleted group had significantly higher levels of serum OVA, collagen and host IgG<sub>2a</sub> specific antibodies than did PBS and isotype control groups, and their draining lymph node cells displayed significantly increased collagen-specific proliferation. Thus an intriguing observation in this model was that depletion of pDC at the later challenge stage of disease appeared to hasten the onset of the antigen-specific adaptive (auto) immune response, while early pDC depletion appeared to slow the onset of adaptive immunity. The specific mechanisms by which these contrasting events appeared to alter the kinetics of disease requires further investigation, but two-photon imaging of the actual cellular interactions occurring *in vivo*, particularly with regard to cognate DC/T cell contact may prove informative.

These experiments proved particularly informative. First, and of clear relevance to all murine models of arthritis, foot pad swelling may not be suitable to use as a sole outcome measurement of pathology, but should be accompanied by histological examination – perhaps the kinetics of swelling and correlation with immune response parameters also requires careful attention. It is also possible that given the onset of swelling on day two and its relatively rapid resolution, the swelling in this instance may have resulted from delayed-type hypersensitivity to the footpad injection. Second, it appears that consistent with other models of arthritis, mDC are capable of eliciting autoimmune disease in the absence of pDC, suggesting that they may constitute the primary DC subset involved in the induction of autoimmunity. Since no specific murine mDC marker has, as yet, been defined, it would be extremely difficult to examine murine pDC elicited autoimmunity in the absence of mDC by a similar method, although our *in vitro* data suggests this unlikely. Third, this model demonstrates that pDC may have a central role in regulating mDC driven autoreactive T cell expansion and autoantibody production. This last observation is consistent with a number of groups reporting that pDC are essential to supporting effective suppression of autoimmunity (700, 703).

The regulation of self-reactive effector responses by specialized populations of regulatory T cells (Tregs) is considered a major mechanism by which tolerance is maintained and autoimmune disease avoided. A number of groups have reported that tolerising pDC in the lymph node are essential for the induction of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, particularly in relation to the prolongation of graft survival in transplant immunology (704, 705). We therefore examined the number of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in the draining lymph nodes from all groups. However, FACS analysis did not identify any significant alterations in the number of Foxp3 expressing T cells between groups. Moreover, we identified comparable CD4<sup>+</sup> expansion in the draining lymph nodes of all groups. Because we were restricted in our examination of Tregs to one time point, the possibility that we missed any differences in this relatively small group cannot be discounted. However, the excessive loss of self-tolerance in the pDC depleted group could potentially be explained by other factors.

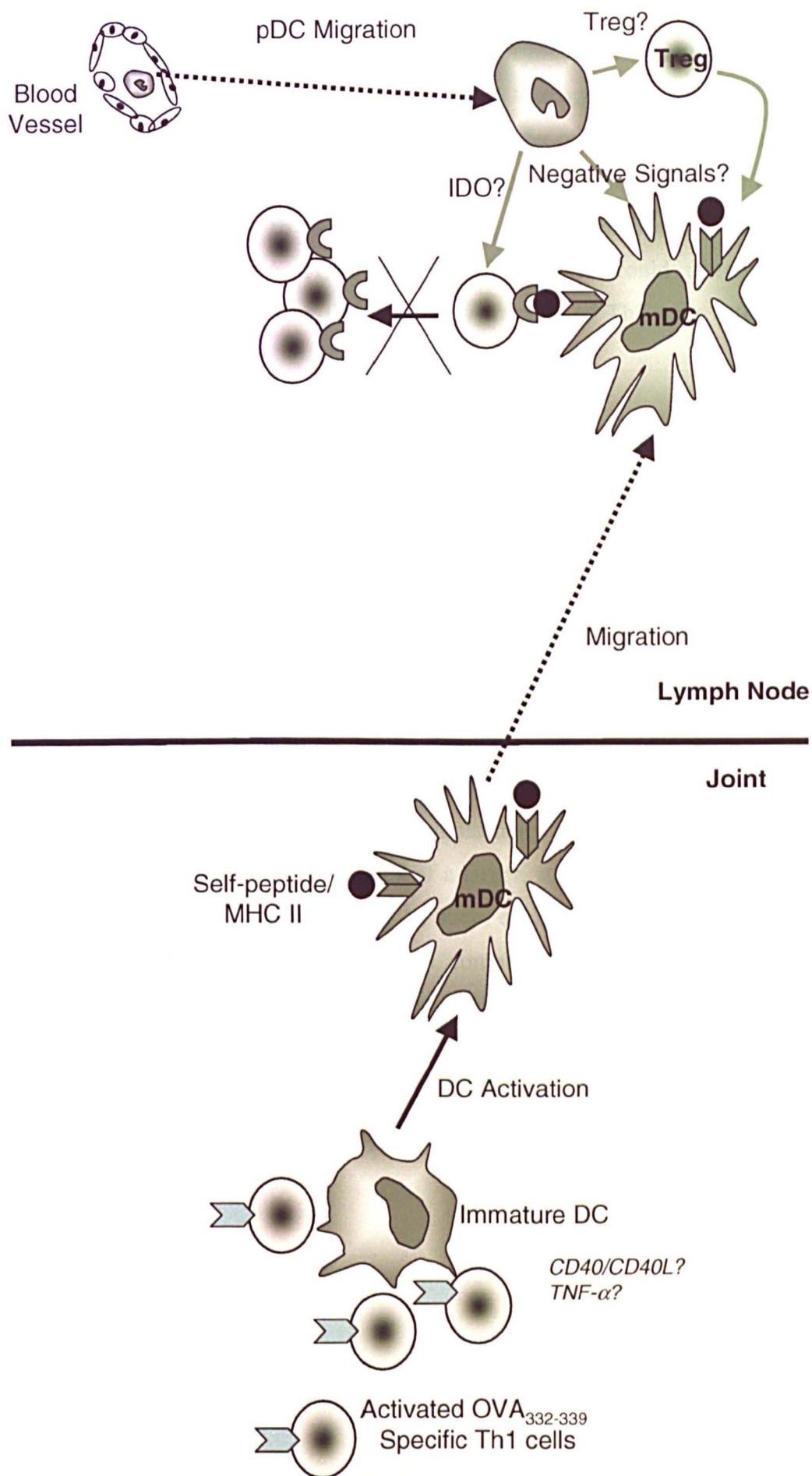
Murine pDC have been credited with an ability to express indoleamine 2,3-dioxygenase (IDO) function (706). IDO is an enzyme that mediates the immunosuppressive pathway of tryptophan catabolism, required during the maintenance of T cell homeostasis and the induction of antigen-specific tolerance (514). Indeed, IDO expressing pDC have been

demonstrated to potently suppress T cell responses to antigens presented by pDC themselves and importantly, in a dominant fashion, antigens presented by immunogenic mDC (707). Furthermore, CTLA-4 Ig engagement of B7 conditions DC to produce IFN- $\gamma$  which promotes IDO induction, thereby initiating degradation of tryptophan to kynurenines in the local tissue microenvironment and concomitant Th1 apoptosis (515). This may explain some of the therapeutic efficacy of CTLA-4 Ig in arthritis beyond the inhibition of APC/T cell contact. Of interest, it has also been shown that engagement of CD200R on pDC, upregulated upon maturation, by soluble CD200-Ig fusion protein elicits IDO enzyme expression and these cells become capable of suppressing Ag-specific responses *in vivo* (708). It is possible in our model that the loss of pDC produced IDO has led to the exacerbation of pathology, but this requires further examination by analysis of serum kynurenine levels, the product of tryptophan catabolism, in depleted and non-depleted animals.

Based upon our investigations thus far, we propose a model whereby tolerogenic mDC patrolling the joint are induced to mature by T cells of irrelevant specificity (in our model OVA TCR Tg T cells) (Fig. 6.17). It is possible that DC maturation and concomitant acquisition of immunogenicity is triggered by OVA-specific cognate interactions with the Th1 polarised OVA TcR Tg T cells. Alternatively, non-specific soluble factors released in the joint in response to intra-articular OVA injection and T cell accumulation may promote DC maturation. Further analysis by two-photon imaging of the joint would resolve this issue. Migration of the immunogenic mDC carrying peptides derived from self (joint)-antigens to the draining lymph nodes could thereafter drive (auto) antigen-specific T cell responses followed by autoantibody production. Since pDC circulate throughout the peripheral blood, migrating directly to the lymph nodes via the high endothelial venules, we propose that at this initial phase of inflammation they retain a predominantly tolerogenic phenotype, allowing them to suppress (although not completely), mDC driven autoreactive T cell expansion. Whether pDC need to present antigen to suppress autoimmunity in an antigen-specific direction in this model is not clear, but again could be resolved by tracking *in vivo*. In addition, the specific mechanisms for disease suppression have not yet been defined and may variously include the expansion of antigen-specific Tregs, the production of IDO, or negative T cell signaling through programmed death receptor ligand-1 (PDCD-1). It is probable that pDC migration to the joint occurs secondary to the initial inflammatory insult in response to up-regulation of

**Figure 6.17 Proposed role of DC subsets in the induction of immunogenicity vs tolerogenicity in the OVA TcR Tg adoptive transfer model of arthritis.**

Following intra-articular OVA challenge, Th1 polarised OVA TcR Tg T cells activate maturation and acquisition of immunogenicity in tissue-sentinel mDC, by the release of soluble factors such as TNF- $\alpha$  and/or cognate interactions (CD40/CD40L). The self-peptide bearing immunogenic mDC migrate to the regional (popliteal) draining lymph node where they induce (auto) antigen-specific expansion of T cells. pDC migrating directly to the lymph node from the peripheral blood may suppress, although not completely, this autoimmune response directly through IDO induced tryptophan catabolism, negative signaling through PD1 or by expansion of antigen-specific Treg.



pro-inflammatory chemokines and synovial angiogenesis. While mDC may be directly pathogenic in triggering inflammatory arthritis, as has been indicated in several murine models, pDC may at this initial phase have more of a tolerogenic role vital to the suppression of autoimmunity. Clearly in this model, the transfer of Th1 cells constituted a significantly severe insult that overwhelmed pDC tolerogenic mechanisms. However, Maffia et al also described a low to moderate transient arthritis induced by adoptive transfer of Th2 differentiated OVA TCR Tg T cells(697). It would therefore be informative to this hypothesis to repeat this model by transfer of Th2 cells into pDC depleted animals.

The observation in this study that depletion of pDC at the time of adoptive T cell transfer reduced disease pathology, whereas depletion at OVA challenge increased disease severity, was intriguing. Although sub-optimal numbers of mice for statistical analysis were used in the early depletion model due to technical restrictions, the observations made may suggest that pDC are necessary at this early stage to enable or enhance disease manifestation in this model. Since pDC are thought to have a life span in murine systems of approximately two weeks (399), it is probable that they were still reduced at the challenge stage, thus the decrease in disease severity could not be accounted for by re-constituted pDC autoimmune suppression. It is more likely that pDC have different functional roles depending upon physiological setting, as presumed by their established plasticity (402). Further analyses of this particular component of the model, in groups of a larger, statistically significant size, will be required in order to confirm the result. As such, elucidating the mechanisms by which pDC depletion induces disease suppression compared to enhancement thereof, particularly in relation to kinetics, may prove particularly informative.

**Chapter 7: General Discussion**



Dendritic cells (DC) are a highly motile heterogeneous network of professional antigen-presenting cell (APC) critical to the initiation and regulation of adaptive immunity, as well as the maintenance of both central and peripheral tolerance. As such, DC biology embodies an important line of investigation in the pursuit of the underlying mechanisms culpable for breach of self-tolerance and generation of chronic autoimmune inflammatory arthritis. However, to date there have been relatively few studies examining the phenotype and function of DC in disease aetiology, particularly with reference to the DC subsets, plasmacytoid (p) DC and myeloid (m) DC. This may be attributed to the complexity inherent in studying a low frequency population of cells, their geographical location in tissues that are not easily accessed and the paucity of specific DC markers available. Accordingly, this thesis utilized novel DC markers in a combination of *in vitro* and *in vivo* analyses in order to elucidate the contribution of different DC subsets to the initiation, modulation and perpetuation of the inflammatory arthritides, rheumatoid arthritis (RA) and psoriatic arthritis (PsA).

The first major component of this thesis sought to examine the profile of human DC subsets in RA and PsA, particularly with regard to phenotype, tissue localization and function in order to gain further insight into the contribution of DC to disease pathology. A number of important observations have subsequently emerged:

- First, we have demonstrated that circulating pDC and mDC are reduced in the peripheral blood (PB) of RA and PsA patients, but are accumulated to the inflammatory synovial fluid (SF) and synovial membrane (SM), commensurate with altered migration.
- Second, we have established that, although mature cells are present, the majority of pDC and mDC within the synovial compartment displayed a predominantly immature phenotype as identified by the low to absent expression of specific co-stimulation and maturation markers including CD80, CD83, CD86 and DC-LAMP.
- Third, we demonstrated that the lack of DC maturation, particularly surprising given the local abundance of TLR stimulating factors such as heat shock proteins and necrotic cellular debris (266), was not a result of an intrinsic functional defect, since SF purified mDC and pDC could mature and release cytokines comparable to their normal PB purified counterparts.

- Fourth, we demonstrated that synovium localized DC also retain functional capacity, expressing the pro-inflammatory cytokines IL-12p70, IL-23p19, IL-15, IL-18 and IFN- $\alpha/\beta$  commensurate with arthritogenicity. Furthermore, mDC and pDC displayed differential cytokine expression. mDC were the predominant IL-12p70 and IL-23p19 expressing cells, commensurate with a central role in the regulation and expansion of T cells, in particular Th17 (625). In contrast, pDC expressed predominantly IL-15 and IL-18, cytokines that can enhance IL-12 induced IFN- $\gamma$  release and inhibit T cell apoptosis (628, 629), thus amplifying and prolonging inflammation. Furthermore, pDC also expressed IFN- $\alpha/\beta$  in abundance. Numerous cases of arthritis triggered by IFN- $\alpha$  therapy of previously non-arthritic patients, which resolved following cessation of treatment, have been reported (543-549), implicating this type I interferon in disease pathology and, by association, the cells which produce it. Indeed, Nestle et al. have recently demonstrated a critical role for pDC derived IFN- $\alpha$  in the early pathogenesis of psoriasis, a defining extra-articular feature of PsA (437). Since pDC exceeded mDC in both RA and PsA synovium, this may have pathological implications commensurate with disease severity and may indicate divergent contributions to disease pathology and, by association, resolution.

In light of the observations gathered thus far, the second component of this thesis sought to examine the effect of cytokine modification of DC function and broader area of synovial inflammation. Specifically, we have examined DC subsets in synovium after TNF- $\alpha$  blockade (infliximab) and thereafter compared and contrasted the effects of both TNF- $\alpha$  and IL-15 on DC subsets in *in vitro* assays. In so doing we sought to elucidate further the contribution of DC to disease pathology and, more specifically, resolution. While this component of the study was hampered by cell rarity, low number of participant patients and the early time point examined after infliximab infusion, it indicated a number of potentially informative trends.

- First, in *in vivo* clinical studies using synovial biopsies obtained before and 48 hours after infliximab infusion, we observed a trend towards decreased mDC, but not pDC, in RA and PsA synovial membrane.
- Second, we established a co-culture system in which cross regulation between T cells and DCs could be examined *in vitro*. These studies demonstrated that TNF

neutralization down-regulated mDC release of the pro-inflammatory chemokines CCL2, CCL4 and CXCL8 following mDC/T cell contact, but not pDC/T cell contact. Indeed, TNF neutralization appeared to *increase* the release of pro-inflammatory chemokines from pDC/T cell contact. A number of groups have suggested that TNF- $\alpha$  may hinder the action and secretion of IFN- $\alpha$  (591) and IFN- $\alpha$  has been demonstrated to drive CCL2, CCL3 and CCL4 secretion from pDC, but not mDC (679). Thus TNF blockade may enable IFN- $\alpha$  activity, and as such enhance pDC arthritogenicity in the longer term.

- Third, using the same *in vitro* system we compared and contrasted these effects of TNF with those of IL-15, an important innate response cytokine heavily implicated in DC effector biology. IL-15 blockade *in vitro* did not increase pro-inflammatory chemokine release from mDC/T cell or pDC/T cell co-cultures, but did decrease TNF- $\alpha$  release in both. The study may therefore suggest the fascinating possibility that TNF-blockade affects distinct DC subsets differentially, which may have therapeutic consequences for patients with a pDC dominant synovial DC infiltrate. As the number of pDC in the inflamed synovium from our patient cohorts undertaking infliximab infusion was low compared to mDC and the majority of patients responded to therapy, we were unable to investigate this hypothesis further. However, it may indicate a point at which IL-15 blockade may complement TNF- $\alpha$  blockade, given the ability of the former to directly inhibit DC/T cell cognate interactions at a point upstream in the inflammatory cascade from TNF- $\alpha$ .

In addition to enhancing pro-inflammatory chemokine release, TNF- $\alpha$  has been demonstrated to limit the maturation, but prolong the life of CD34<sup>+</sup> progenitor cultured DC (655, 656). Thus the local abundance of TNF- $\alpha$  in the inflamed synovium may be considered a candidate extrinsic factor facilitating the accumulation of immature DC to the inflamed synovial compartment. As such, immature DC in the synovium could be '*frustrated*' by their inability to mature. However, the identification of a small population of CD83<sup>+</sup> and DC-LAMP<sup>+</sup> mDC and pDC may indicate that some DC can escape this mechanism. Indeed, the pattern of immature to mature pDC and mDC infiltration to the SM may be indicative of a local maturation gradient, commensurate with DC migration to, and maturation within, the synovium. Given the abundance of self (auto) antigen in the SF, if mature SM localised DC originate from the pool of immature SF DC, there is

potential for *in situ* presentation of arthritogenic peptide to autoreactive T cells. Indeed, both pDC and mDC within the SM could be co-localised to CD4<sup>+</sup> and CD8<sup>+</sup> T cell aggregates commensurate with cell contact. However, while a number of studies have identified oligoclonal CD4<sup>+</sup> and CD8<sup>+</sup> T cells in RA and PsA SM, it is unclear whether clonal expansion occurs *in situ* or in the regional lymph node with attendant remigration to the tissue thereafter. Studies ongoing in our laboratories are directly addressing this question using two-photon microscopy to track antigen activated T cells *in vivo*.

One problem with such studies had been until now the lack of good arthritis model systems in which to address such issues. However, we were able to take advantage of a recently developed antigen driven arthritis model generated in our laboratory to evaluate the role of DC subsets in evolving articular inflammation. Comparative analysis of the function of DC subsets in the OVA TCR murine model of arthritis demonstrated that pDC in particular, may be vital to the *suppression* of autoimmunity by the maintenance of peripheral tolerance. Adoptive transfer of Th1 polarized OVA-specific TcR Tg T cells to BALB/c recipients produces arthritis in joints proximal to the site of OVA inoculation, (presumably) by triggering immunogenic maturation of tissue circulating, self-peptide bearing DC and consequently auto-reactive T cell expansion (697). We demonstrated that depletion of pDC in this model significantly increased disease severity, as ascertained by histology, serum auto-antibodies and (self) antigen-specific CD4<sup>+</sup> memory T cell proliferation, thus suggesting a regulatory role for pDC in autoimmune suppression. Moreover, this model demonstrated that mDC can instigate autoimmunity in the absence of pDC. Indeed, *in vitro* examination of the collagen type II processing and presentation capacity of murine bone marrow differentiated mDC and pDC established that while both subsets could induce antigen-specific memory CD4<sup>+</sup> expansion, only mDC elicited expansion of (presumably) naïve autoreactive T cells. Together these data imply that mDC may constitute the dominant DC subset culpable for eliciting arthritogenic autoimmunity, whereas pDC may be vital to its suppression, at least during the expansion or initiation phase of the disease process. However, the circumstantial human studies contained in this thesis clearly indicate that both mDC and pDC exhibit the potential to be pathologic within the synovial compartment during the chronic phase of arthritic inflammation, not least via cytokine release.

The presence, or absence, of tolerogenic DC in the inflamed synovial compartment in human disease has not yet been established. Of interest, TNF- $\alpha$  has been shown to induce tolerogenic DC, as defined by a semi-mature phenotype, capable of inducing antigen-specific protection from autoimmunity in a number of murine models (709, 710). Commensurate with this, we observed that mDC and pDC within both RA and PsA SF displayed partial upregulation of maturation receptors, potentially indicative of a semi-mature/tolerogenic phenotype. Furthermore SF, but not healthy donor PB, purified mDC and pDC released IL-10, a major defining functional feature of tolerogenicity. In the steady-state, DC maintenance of peripheral tolerance by induction of antigen-specific regulatory T cells (Treg) is perceived to occur in the regional lymph node. While CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg have been identified in RA SF and synovium (711, 712), it has not yet been established if Treg migrate to, or differentiate within, the synovial compartment. In any event IL-15, which is dispersed throughout the synovial compartment and expressed by both pDC and mDC, has been shown to abrogate the suppressive activity of Treg (713). As such, IL-15 blockade in disease may release Treg modulation of aberrant immunity. It is clear however, that in the context of chronic autoimmunity, self-tolerance is broken and the mechanisms that would normally re-establish it overwhelmed.

IL-15 blockade currently constitutes a promising biological therapy in inflammatory arthritis (341). We therefore sought to elucidate the broader biological mechanisms by which IL-15 neutralisation could operate, particularly as it was expressed by both pDC and mDC within the inflamed synovium. Specifically, we sought to characterize points by which IL-15 neutralisation may hinder T cell cognate interactions with DC.

- First, we demonstrated that neutralization of exogenous IL-15 suppressed proliferation and induced apoptosis of an IL-15 sensitive cell-line.
- Second, we established that IL-15 blockade neutralised exogenous IL-12/IL-15 induced IFN- $\gamma$  release from SF derived mononuclear cells (SFMC), thus confirming its bioactivity. Moreover, we demonstrated that IL-15 blockade neutralised not only induced, but also spontaneous TNF- $\alpha$  release from SFMC, thus confirming its role upstream from TNF- $\alpha$  in the inflammatory cytokine cascade.
- Third, we confirmed effective neutralization of endogenous IL-15 from SF purified leukocytes by demonstrating IL-15 blockade induced down-regulation of CD69 on

SF derived T cells. Since CD69 is implicated in cognate interactions with APC (630), this may therefore indicate a mechanism by which IL-15 neutralization could inhibit cognate DC/T cell contact. In addition, we demonstrated that IL-15 neutralising antibody could bind IL-15 at the cell surface thus inhibiting juxtacrine IL-15 interactions.

The critical challenge in therapeutics now is to re-establish tolerance, such that therapeutic remission can be maintained. It has now been demonstrated by a number of studies that combination therapy using methotrexate and TNF-blockade, early in disease during a so-called “window of opportunity”, induces significant, sustained remission in RA patients even after withdrawal of TNF-blockade (714, 715). It is likely that the relative success of early intervention is possible by halting disease progression prior to establishment of the amplifying feedback loop between (adaptive) autoimmune cartilage / bone destruction and (innate) TLR stimulation. But what of tolerance induction in patients suffering long-term destructive disease? Promisingly, rituximab (anti-CD20) depletion of B cells from patients with active RA has been shown capable of inducing extended periods of disease remission in about 50% of patients, sustained for up to one year after rituximab infusion (290). However, as with TNF-blockade, B cell depletion is effective in only a proportion of patients. A possible explanation may be that these therapies target specific arms of the immune system, which may prove successful in (for example) genetically susceptible patients, but fails patients in whom both innate and adaptive immunity play a collaborative, destructive role. Moreover, these patients may not be ‘immunologically tolerant’, but the force of such studies is the indication that long term manipulation of inflammatory responses is feasible and that given the appropriate reagents and clinical intervention strategy, it may be possible to truly modify disease.

Optimal adaptive immunity requires induction by innate immune effector and regulatory pathways in the initiation and effector phase of arthritis. This is demonstrated in the K/BxN murine model of arthritis, whereby pathological activation of macrophages, neutrophils and mast cells is triggered by immune complexes formed by autoantibody against the ubiquitously expressed protein glucose-6-phosphate isomerase (GPI) (322-325). However, the production of anti-GPI antibodies requires complement C5, as C5-deficient mice do not develop disease. In humans, only a very low association has been made between arthritis and the presence of auto-antibodies to GPI (308, 315). Conversely,

numerous studies have identified the presence of anti-CCP antibodies, identifiable in circulation as early as 10 years before the onset of clinical disease, as a major risk factor in the development of RA and severe PsA. Moreover, an interrelated association has been described between anti-CCP antibodies, cigarette smoking and HLA-DR shared epitope expression, inferring involvement of both innate and adaptive immunity to the underlying disease pathology. As DC are *the* immune cells inducing innate immunity and conducting adaptive immunity and, as has been demonstrated in this study, may be important not only to the initiation and prolongation of chronic arthritis but also the suppression thereof, DC may constitute promising therapeutic targets in the 'pre-clinical disease' phase of rheumatologic autoimmunity. Of note, while inhibition of DC pro-inflammatory functions may have immediate benefit, the next challenge may be to uncover mechanisms by which DC tolerogenicity can be "released" and immune tolerance "re-set". Of note, CTLA-4Ig infusion, which has demonstrated moderate therapeutic success supposedly by inhibition of APC/T cell contact, also induces pDC to release IDO, thus enhancing tryptophan catabolism and T cell apoptosis (515). Thus re-establishing mechanisms of immune suppression may be possible. Furthermore, adoptive transfer of tolerogenic DC has been shown to induce disease amelioration in murine models of arthritis (716).

The current study addressing as it does a rare cell population functioning in difficult tissue locations, has by necessity a number of weaknesses. In particular, it was difficult to establish whether pDC and mDC from the synovial compartment were hyper-responsive to TLR stimulation, which has been implied by other studies analyzing monocyte-derived DC (614), or indeed if they were hypo-responsive, which may be further indication of a tolerogenic phenotype. The difficulty was largely attributable to low useable sample numbers and the technical difficulties inherent in purifying any cell, let alone rare ones, from SF. Furthermore, due to the decreased PB mDC and pDC in RA and PsA, the function of these could not be investigated at all due to ethical restrictions. In future, novel analyses by which the function of very rare PB DC can be investigated will be required. Investigation of the IL-10 expression, or lack thereof, by mDC and pDC within synovium will be required to determine whether membrane DC represent a heterogeneous population of arthritogenic and tolerogenic DC. A further weakness of the study was that the effect of TNF-blockade on PB circulating mDC and pDC was not undertaken due to clinical trial prohibition. While this has been attempted by one other group (680), they did not use specific DC markers, thus the results are difficult to interpret. In light of our observation

that TNF- $\alpha$  affects DC subsets differentially, such an analysis in future, particularly using the sensitive assay system described in chapter 3, may be informative. Finally, although this study has attempted to examine the cognate interactions between mDC, pDC and T cells, it has not addressed DC interactions with B cells. Since recent studies have indicated that DC, in particular pDC, may interact with B cells directly, inducing isotype class switching (399, 717, 718), such investigations will be required to elucidate the potential direct contribution of DC to autoantibody production.

In summary, the present thesis represents a parallel design clinical, *in vitro* and *ex vivo* analysis of the role played by DC subsets in inflammatory arthritis. It has defined for the first time the definitive phenotype of a variety of DC subsets in RA and PsA. Inevitably it has raised a new set of questions and has only partially answered those questions present at outset. A number of studies are now urgently required.

- First, *ex vivo* analysis of circulating pDC and mDC subsets pre- and post-TNF blockade should be performed. By undertaking these experiments it is hoped to elucidate further the differential effects of TNF- $\alpha$  on the DC subsets.
- Second, commensurate with the above, analysis of type I IFN expression in SM taken before and after TNF blockade, particularly as related to clinical response measures, could potentially give further insight into why certain patients fail TNF- $\alpha$  blockade.
- Third, while we co-localised mDC and pDC to T cell aggregates in the inflamed synovium, we did not analyse their distribution in relation to germinal centres. Considering that pDC in particular have been demonstrated to interact directly with B cells, inducing IgG class switching (717, 718), this could prove informative.
- Fourth, given the predominant immature phenotype of SF residing mDC and pDC, and their concomitant potential for self (auto) antigen presentation, analysis of mDC and pDC infiltration to the local cartilage would be enlightening.
- Fifth, in order to expand our understanding of the role of pDC in the murine model of arthritis presented here, depletion of pDC in an adoptive transfer model using Th2, as opposed to Th1, polarized OVA specific T cells is required. Maffia et al have previously reported that transfer of Th2 polarised OVA specific Tg T cells induces only a very mild transient arthritis in recipient BALB/c (697). If pDC suppress autoimmunity in a 'blanket' manner, it would be expected that their



depletion in Th2 transfer would instigate more severe pathology. Alternatively, if Th2 transfer drives pDC function towards a more arthritogenic as opposed to tolerogenic functional phenotype, particularly given that pDC were originally defined as ‘DC2’ – Th2 polarising DC, the reverse would be expected.

- Finally two photon imaging of the joint during this arthritis model would allow us to determine whether OVA TcR Tg T cell cognate interactions induce immunogenicity in the tissue sentinel DC, or whether this pivotal point in the breach of self-tolerance is driven by non-specific soluble factors released during inflammation.

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## Publications

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3. Lebre MC\*, Jongbloed SL\*, Tas SW, Reinders-Blankert P, Smets TJM, McInnes IB, Tak PP: **Rheumatoid arthritis synovium contains immature myeloid and plasmacytoid dendritic cells with distinct cytokine profiles**  
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4. Gracie JA, Jongbloed SL, Wilson H, McInnes IB: Innate response cytokines in inflammatory synovitis: interleukin-15 and interleukin-18, in: **Cytokines and Joint Injury**, van den Berg WB and Miossec P (eds), Birkhauser, Berlin (2004): 127-145.